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Influence of nitrogen on fungal communities involved in mobilization of nutrients from primary minerals in forest soil

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ABSTRACT

N input to forest ecosystems is likely to affect soil microbial communities and biogeochemical weathering processes that lead to mobilisation of mineral nutrients essential for ecosystem functioning. The objective of this study was to test whether N addition affects mobilisation of mineral nutrients by ectomycorrhizal fungi in a forest soil amended with different primary minerals. Mor-layer soil with extensive ectomycorrhizal colonisation was collected from a mixed Pine-Spruce-Aspen forest at Lunsen, Uppsala, Sweden and used as substrate in a laboratory microcosm experiment. Pine seedlings were grown in this substrate after amendments with two concentrations of a slow release N fertiliser and different primary minerals (quartz, apatite and biotite) in factorial combinations. Basidiomycete community profiles from soil samples were examined by denaturing gradient gel electrophoresis (DGGE) and elemental analyses of soil solutions and plants were performed. Seedlings grown in apatite-amended substrates had significantly higher biomass compared to seedlings grown in biotite-treated substrates and also had substantially higher P concentrations. N appeared to have a negative effect on mobilization of nutrients from these minerals, and to increase P deficiency in the soil solution in quartz and biotite treatments. However this negative effect was less evident for the rest of the nutrients. In general, N improved ectomycorrhizal abundance in the soil and, similar effect was observed in apatite treatment alone.

Keywords: apatite, biotite, denaturing gradient gel electrophoresis, ectomycorrhiza, nitrogen fertilization, weathering.

RESUMO

Influências antropogénicas na entrada de azoto em ecossistemas florestais podem influenciar as comunidades microbianas do solo e os processos de erosão biogeoquímicos que levam à mobilização de nutrientes minerais essenciais ao funcionamento do ecossistema. Esta experiência teve como objectivo estudar o efeito da adição de N sobre as comunidades fúngicas de um solo florestal, em particular os fungos ectomicorrízicos, envolvidos na mobilização de nutrientes a partir de minerais primários. A camada orgânica mor depositada sobre afloramentos rochosos graníticos em uma floresta de coníferas em Lunsen, Uppsala, foi usada como substrato numa experiência em microcosmo juntamente com plântulas de pinheiro-silvestre. O solo recolhido foi misturado com quartzo, apatite e biotite juntamente com um fertilizante de libertação lenta em diferentes níveis. Os perfis da comunidade Basidiomycota foram obtidos através de electroforese em gel com gradiente desnaturante e a análise química dos elementos do solo e planta foi obtida. Plantas em solo com apatite cresceram significativamente melhor comparado com biotite, apresentando um aumento substancial nas concentrações de fósforo. Aparentemente o azoto teve um efeito negativo na mobilização de nutrientes a partir dos minerais e, à excepção do tratamento com apatite, houve intensificação na deficiência em fósforo. A adição de azoto aumentou a abundância de fungos ectomicorrizicos no solo e, o mesmo efeito foi observado na adição de apatite.

Palavras-chave: apatite, biotite, electroforese em gel com gradiente desnaturante, ectomicorrizas, fertilização de azoto, mobilização.

RESUMO ALARGADO

As influências antropogénicas na entrada de azoto em ecossistemas florestais podem influenciar as comunidades microbianas do solo e os processos de erosão biogeoquímicos que levam à mobilização de nutrientes minerais essenciais ao funcionamento do ecossistema. Esta experiência teve como objectivo estudar o efeito da adição de N sobre as comunidades fúngicas de um solo florestal, em particular os fungos ectomicorrízicos, envolvidos na mobilização de nutrientes a partir de minerais primários. Pretendeu-se testar a hipótese de que a aplicação de N reduz a abundância relativa e diversidade de espécies de fungos ECM com a capacidade de mobilizar nutrientes a partir de minerais primários. A camada orgânica mor depositada sobre afloramentos rochosos graníticos em florestas de coníferas em Lunsen, Uppsala, foi usada como substrato numa experiência em microcosmo juntamente com plântulas de pinheiro-silvestre. O solo recolhido foi misturado com quartzo, apatite e biotite juntamente com um fertilizante de libertação lenta em dois níveis de intensidade, alto nível de N e baixo nível de N. Os perfis da comunidade Basidiomycota foi obtida através de métodos moleculares, nomeadamente PCR (polymerase chain reaction), que permitiu a amplificação do DNA de Basidiomycotas presente no solo e, electroforese em gel com gradiente desnaturante (DGGE) que permitiu detectar alterações na composição da comunidade fúngica ao longo do tempo. A análise química dos elementos do solo e das plantas foi obtida, de modo a se poder inferir sobre a mobilização e absorção de nutrientes pelas plantas, derivados dos minerais. Plantas em substrato com adição de apatite cresceram significativamente melhor comparado com adição de biotite, apresentando um aumento substancial nas concentrações de fósforo e uma intensificação da acidificação do solo. No entanto as plantas apresentaram um reduzido nível de micorrização comparado com os restantes tratamentos. Aparentemente o N teve um efeito negativo na mobilização de nutrientes a partir dos minerais pois verificou-se a redução de catiões presentes na solução do solo juntamente com intensificação de deficiência em fósforo em todos os tratamentos à excepção do tratamento com apatite. Os perfis da comunidade fúngica presente no solo, sob diferentes tratamentos permitiram observar uma modificação na composição de bandas, especialmente em função da aplicação de N. As bandas sequenciadas forneceram identidade ao nível da espécie, na sua maioria, o que permitiu concluir sobre a elevada importância dos fungos ectomicorrizicos neste tipo de solos. Parece ter havido selecção de certos fungos ectomicorrizicos em função da aplicação de azoto no solo. O tratamento com apatite mostrou um padrão diferente dos restantes, pois o efeito do N foi menos evidente, uma vez que as bandas geralmente ausentes nos tratamentos sem N pertencentes ao controlo, quartzo e biotite encontravam-se presentes no tratamento sem N com adição de apatite.

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LIST OF ABBREVIATIONS

ANOVA – analysis of variance

APS - Ammonium persulfate

bp – base pairs

CTAB - Hexadecyltrimethylammonium bromide

ddH_2O - double-distilled water

DGGE – Denaturing gel gradient electrophoresis

dNTPs - deoxynucleosides

DNA – Deoxyribonucleic acid

dsDNA - double-stranded DNA

ECM – Ectomycorrhizal

FIA - Flow Injection Analyzer

GC – Guanine - Citosine

HSD - Honestly significant difference

ICP-AES - Inductively Coupled Plasma-Atomic Emission Spectroscopy

ITS – Internal transcribed spacer

PAR - Photosynthetically active radiation

PCR – Polymerase chain reaction

LMWOAs - Low molecular weight organic acids

LSU - large subunit

rRNA - ribosomal RNA

SIP – Stable isotope probing

SSU - small subunit

TAE buffer - Tris-acetate EDTA buffer

TMED - N,N,N',N'-Tetramethylethylenediamine

1 BACKGROUND

1.1 NITROGEN FERTILIZATION IN BOREAL FORESTS

Nitrogen (N) is considered the most limiting nutrient in boreal forest systems (Vitousek and Howarth, 1991; Wallenda and Kottke, 1998; Allison et al., 2008), affecting not only plant growth (Barbour et al., 1987; Nygren, 2008) but all the biota (Vitousek and Howarth, 1991). Fossil fuel combustion and use of N fertilizers are the main anthropogenic sources of N (Koehler et al., 2009), resulting in enhanced emissions into the atmosphere during the recent decades, and promoting deposition and amounts of reactive forms of N in ecosystems (Galloway et al., 2003; Enowashu et al., 2009). Nitrogen deposition is likely to remain high in the future due to climate warming (Allison et al., 2009), although anthropogenic N deposition in boreal forests is small when compared to the rest of the globe (Allison et al., 2007).

Increases in temperature, as predicted in high latitudes as a result of global warming, will increase the rate of decomposition of soil organic matter by enhancing microbial activity (Vitousek and Howarth, 1991). This can contribute positively to soil nutrient availability and plant productivity in northern ecosystems (Manninen et al., 2009). Such changes could then feedback to have additional effects on plant and microbial communities (Allison et al., 2009). In northern forest ecosystems, most of the soil N is in organic forms and becomes less available during the transition from mineral nutrients to living and dead microbial tissue to plant litter and finally to humified material (Tamm, 1991). Forest trees have a limited ability to utilise organic N sources although they can use simple amino acids when these are available (Näsholm and Persson, 2001).

The boreal forest is the largest terrestrial biome on earth, representing 33% of total forest cover (FAO, 2001). It spreads over Russia, Canada, USA, Sweden, Finland and Norway (Bradshaw et al., 2009). This biome is mainly characterized by coniferous forests dominated by slow-growing trees and an understorey of ericaceous shrubs (Manninen et al., 2009). The climate is characterized by pronounced seasonal variations from short warm summers to long and extremely cold winters. These cold temperatures restrict the growing season to a relatively short period (Bonan, 1989), and both evapotranspiration and decomposition rates are low, resulting in the accumulation of plant organic residues either as raw materials at the soil surface or as peat (Rosling et al., 2003; Read et al., 2004). Fallen leaves, needles and moss can remain relatively intact on the forest floor for long periods of time (Bonan, 1989). Boreal soils are usually young, acidic (Ruckstuhl et al., 2008) and poor in nutrients (Nygren, 2008). Since tree growth is often limited by the

small availability of soil N in boreal systems (Tamm, 1991; Vitousek and Howarth, 1991), N fertilization is a common practice used to improve forest productivity.

In Sweden, mainly in central and northern regions, large-scale N fertilization began in the mid-1960s, when foresters became aware of the potential effects of N fertilization on tree growth, especially in coniferous trees such as Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* L.). Initially, during the late 1970s, nearly 200 000 ha were fertilized annually compared to the current annual fertilization of 30 000 ha (Nohrstedt, 2001). Forest fertilization aims to increase wood production by improving the nutrient supply in poor soils, balancing the soil nutrient status and reducing soil acidity (best achieved by slow-release fertilizers) (Saarsalmi and Mälkönen, 2001). Nitrogen alone promotes stem growth of most coniferous trees, and is the only nutrient that has been applied in commercial stands (Nohrstedt, 2001). Consequently, N fertilization is considered a cost-effective practice to increase forest yields (Nohrstedt, 2001; Saarsalmi and Mälkönen, 2001) in intensive forest management. However, we need to take into account the fact that boreal ecosystems can be especially sensitive to an increase in nutrient supply (Manninen et al., 2009), for example from N fertilization, since they may be adapted to low nutrient availability due to slow rates of mineralization (Read, 1991).

1.2 ECTOMYCORRHIZAL FUNGI

1.2.1 Mycorrhizal fungi and ectomycorrhizal symbiosis

Fungi play an important role in the release and acquisition of nutrients from soil organic matter in boreal forests, and consequently influencing soil fertility and nutrient cycling in these ecosystems (Allison et al., 2007, 2009; Högberg et al., 2007; Lindahl et al., 2007; Finlay, 2008). Fungi are favoured over the rest of the microbial community due to the acidic nature of most soils with a recalcitrant litter layer (Allison et al., 2007), the seasonality of climate with low temperatures and surface drying (Read, 1991). Therefore, they represent a large proportion of the active biomass in boreal forest ecosystems (Rosling, 2003), which in turn influences the establishment, health, survival and decomposition of forest trees.

As heterotrophic organisms, fungi use external carbon sources, combining extracellular digestion and absorption (Webster and Weber, 2007). They adopted different trophic strategies to obtain carbon: saprotrophs, necrotrophs and biotrophs (Finlay, 2008). According to Read and Perez-Moreno (2003), in environments with limited mineralization that could threaten the fitness of autotrophs, selection has favoured symbiotic fungal

associations that enable the capture of limiting nutrients, such as N, from organic sources.

Mycorrhizal fungi are specialized soil fungi that form symbiotic associations with plant roots (Smith and Read, 2008). The symbiosis was first described by Frank in 1885. Mycorrhizas play a central role in nutrient uptake by plants and are the most ancient form of fungal symbiosis with plants. It has been suggested that these symbiotic associations were a necessary step in the colonization of land by plants, accessing nutrients that were otherwise inaccessible to primitive root systems (Smith and Read, 2008).

Seven types of mycorrhizal symbiosis have been distinguished according to their morphological characteristics and the fungal and plant species involved (Smith and Read, 2008). Finlay (2005; 2008) reviewed the different mycorrhizal categories. *Arbuscular mycorrhizas* are known to be the most ancient and widespread form of mycorrhizal symbiosis, where fungi belonging to the Glomeromycota form symbioses with a wide range of plant species. The symbiosis is characterized by highly branched fungal structures, arbuscules, which grow intracellularly without penetrating the host plasmalemma. *Ericoid mycorrhizas* are formed in three plant families, all belonging to the order Ericales, with various fungi from the Ascomycota. These plants grow mostly in upland and lowland heaths and other nutrient-impoverished areas, such as the understorey vegetation of boreal forests and chaparral vegetation systems, in warm Mediterranean climate zones. The fungus penetrates the cell walls of roots and forms coiled structures within each cell without penetrating the host plasmalemma. Orchids form *orchid mycorrhizas* with a range of basidiomycete fungi. Because some orchid species are achlorophyllous as adults, the germination and early development phase are dependent on an external supply of nutrients and organic carbon. These plant species are usually described as 'myco-heterotrophic', obtaining their carbon from fungi.

A fourth type of mycorrhiza is also formed by myco-heterotrophic plants, from the family Monotropaceae, *monotropoid mycorrhiza*. This symbiosis is structurally similar to ectomycorrhizas, characterized by a well-developed fungal mantle and a more superficial Hartig net, with single hyphae growing into the epidermal cells, but not into the underlying cortex, forming structures known as fungal pegs. The achlorophyllous monotropoid plants are fully dependent on the fungi for soil nutrients and carbon obtained indirectly from autotrophic host plants occurring in the vicinity, which are also attached to the same ectomycorrhizal mycelium (Leake et al., 2004). Many fungi that are normally ectomycorrhizal, when colonizing plants in the genera *Arbutus* and *Arctostaphylos* and the family Pyrolaceae, form *arbutoid mycorrhizas.* In this form of symbiosis, mantle and Hartig net structures are also present but extensive intracellular

fungal proliferation also occurs. Another mycorrhizal symbiosis is the *ectendomycorrhizas*. These are characterized by features of both ectomycorrhizas and endomycorrhizas, a fungal sheath that may be reduced or absent, a Hartig net usually well developed, but also intracellular penetration.

Lastly, *ectomycorrhizal* (ECM) fungi are the most common symbiosis in boreal forests trees (Wallenda and Kottke, 1998; Read et al., 2004) and are predominantly from the Basidiomycota and, to a lesser extent, Ascomycota, exhibiting a large species richness with as many as 7 – 10 000 fungal species colonising about 8 000 plant species (Taylor and Alexander, 2005). The plants involved are usually woody perennial trees and shrubs from a wide variety of ecosystems (Finlay, 2008) with a global importance since they occupy a disproportionately large fraction of the total terrestrial land area in relation to their species numbers and have economic value for timber production. An example is the family Pinaceae, a significant representative of boreal forests of the northern hemisphere (Smith and Read, 2008). The ECM fungi form a structure called the mantle, where a mycelial sheath is developed around each of the short lateral roots. Hyphae also penetrate between the epidermal and cortical cells, forming a network of intercellular hyphae, the Hartig net (Peterson et al., 2004). The mantle is extended into a more or less well developed extraradical mycelium into the soil (Finlay, 2008), forming an important connection with ECM sporocarps (Smith and Read, 2008) and constituting an important fraction of the total microbial biomass present in forest soils (Wallander et al., 2001). These ECM soil-borne mycelia are functionally important for the symbiosis, being directly involved in mobilization and uptake of nutrients and water from the soil (Smith and Read, 2008) and interacting with organic and inorganic substrates (Finlay and Rosling, 2006). In return, the autotrophic plant host provides carbohydrates *via* photosynthesis to the heterotrophic fungus (Wallenda and Kottke, 1998). The Hartig net in the ECM root is critical to the symbiosis since it is where the transfer of nutrients and carbon between the partners takes place (Anderson and Cairney, 2007; Smith and Read, 2008).

In boreal ecosystems, biotic interfaces with soil organic matter and minerals, which are dominated by the mycelial systems of mycorrhizal fungi rather than by the roots themselves, are the main suppliers of plant nutrients (Read et al., 2004). Even though there is an abundance of organic matter in many forest ecosystems, nutrients are often in complex organic forms that are unavailable for direct plant uptake and ECM fungi can access the N and P in these more recalcitrant pools and provide their host plants with nutrients (Allen et al., 2003).

1.2.2 Ectomycorrhizal mats

Mor type humus is described by Green et al. (1993) as generally consisting entirely of organic horizons with acidic organic materials and high C:N ratios, suggesting storage of nutrients which are slowly released and made available to plants. Organic matter accumulates on the soil surface and compact matted horizons reflect the slow decomposition processes taking place, with fungi being the dominant decomposers. It is usually developed where climatic and/or edaphic conditions are unfavorable for the development of more biologically active humus. Taylor et al. (2009) pointed out that ectomycorrhizas are increasingly associated with the process of humus accumulation in soil because of their ability to absorb N in organic and inorganic forms, as well as their low expression of cellulose or lignin degrading enzymes which results in a large accumulation of recalcitrant organic rich "mor" humus.

Various ECM fungi are known to form dense aggregations of hyphae known as mats (Griffiths et al., 1990; Agerer, 2001). These fungal mats can be formed in both the mineral and organic horizons of forests, with a significant impact on the biological, chemical, and physical properties of the soils they occupy (Kluber, 2010). They have been considered to be good model systems to study the effects of ectomycorrhizas on soil processes (Cromack et al., 1979; Griffiths et al., 1990), because of their ability to dominate the soils locally (Kluber et al., 2010). Soils colonized by ECM fungal mats usually differ from non-mat forest soils, with greater microbial biomass, with the fungal rhizomorphs representing up to half of the dry weight of the mat-associated soil (Ingham et al., 1991). A large content of organic matter is usually present, together with elevated levels of oxalate and a lower pH (Griffiths et al., 1994; Malajczuk and Cromack, 1982). Previous studies showed that ECM mats can increase enzymatic activities and litter decay rates (Entry et al., 1991; Griffiths and Caldwell, 1992), provide habitat for soil animals (Cromack et al., 1988), possibly enhance seedling survival (Griffiths et al., 1991) and accelerate mineral weathering (Cromack et al., 1979; Griffiths et al., 1994). In particular, the oxalic acid and calcium oxalate produced by various ECM fungi (Cromack et al., 1979; Malajczuk and Cromack, 1982; Lapeyrie et al., 1987) are thought to enhance weathering of soil minerals (Griffiths et al., 1994). However, the low values of pH are not necessarily restricted to soils with extreme hyphal densities, as tree species can also influence soil pH (Taylor et al., 2009).

1.2.3 Effect of nitrogen on ECM communities

Due to limited N availability in boreal ecosystems, inorganic N fertilizers have been widely used, changing the nutrient cycle in soils and consequently nutrient availability to trees (Fransson et al., 2000). This may have repercussions on the microbial community present in the soil. It is in the surface layer of the soils that ECM roots of boreal trees are concentrated, intimately associated with the organic matter (Read, 1991). Boreal forest trees are thought to be largely dependent on ECM fungi for N uptake, especially with respect to N acquisition from organic sources, because they are able to sequester N from organic polymers (Read and Perez-Moreno, 2003; Nygren et al., 2007). ECM fungi are also sensitive to N (Wallenda and Kottke, 1998), and N availability in the soil can then influence mycorrhizal abundance (Treseder, 2004). Consequently, it has been hypothesized that when small amounts of N are available plants invest more carbon in mycorrhizal fungi since they can contribute to nutrient uptake by plants (Mosse and Phillips, 1971). Conversely, plants are thought to reduce carbon allocation to mycorrhizal symbionts when N is more readily available (Högberg et al., 2003; Nilsson and Wallander, 2003; Treseder et al., 2007) allocating carbohydrates (and N) to shoot growth which may result in decreased mycorrhizal abundance (Read, 1991).

In many forest fertilization studies, species composition and richness have been reported to change with N application (Allison et al., 2007; Demoling et al., 2008; Nygren, 2008). According to Fransson et al., (2000) the effects of fertilization on the ectomycorrhizal structures may depend on the type of fertilizer used and the application method, for example whether the fertilizers are applied in large single doses or in continuous application of smaller doses. Nohrstedt (2001) reviewed the effects of N fertilization based on Swedish experiments. Production of mycorrhizal sporocarps appears to be especially sensitive to regular N inputs (annual addition during an extended period) but also to single doses. However, the durability of the effects has been reported to last just a few years. Colonized root tips seem to be less affected, their abundance being temporarily reduced after a single dose application that will recover after just a couple of years (Arnebrant, 1991). However, some changes in species richness and composition may occur, favouring N-tolerant species (Nilsson and Wallander, 2003). The production of external mycelium is often reduced in response to N addition in laboratory (Wallender and Nylund, 1992; Arnebrant, 1994) and field studies (Nilsson and Wallander, 2003).

1.3 WEATHERING OF PRIMARY MINERALS

Soil mineral weathering, together with atmospheric deposition, is one of the main sources of nutrients (except for N and C) and is necessary for the long-term sustainable growth of most forest ecosystems (Hoffland et al., 2004; Courty et al., 2010). Granites and gneisses make up the majority of Swedish bedrocks and contain primary minerals such as quartz, feldspar, mica and apatite (Rosling, 2003). Several physical, chemical and biological processes control mineral weathering (Courty et al., 2010). Due to their instability under earth conditions (Banfield et al., 1999), where mechanical erosion occurs but also thermal and hydration effects, elements such as phosphorus, calcium, potassium, magnesium, and many trace elements are released in forms that are directly available to the biota (Hoffland et al., 2004). These elements can also recombine to form secondary minerals, such as clays and oxides that are much more stable under current environmental conditions (Rosling, 2003). The dissolution rate is also influenced by their nature and the active surface area (derived from particle size and soil moisture) but also by chemical reactions in the soil solution (Finlay et al., 2009). These are dependent of the chemical composition of the soil solution, which in turn is also strongly correlated with the concentration of mineral weathering agents such as protons, organic and inorganic acids and siderophores (Watteau and Berthelin, 1994; Barker et al., 1997). Biological factors have also been acknowledged to contribute to mineral dissolution (Finlay et al., 2009). Plants can enhance mineral weathering by releasing organic compounds, increased contact area between biological material and mineral surfaces, a longer water residence time (Drever, 1994; Barker et al., 1997) and nutrient uptake (Finlay et al., 2009; Courty et al., 2010). Microorganisms colonizing mineral surfaces have also been documented (Banfield et al., 1999; Gorbushina, 2007) as well as their effect on the stability of minerals through the production of various metabolites (protons, acids, siderophores, polysaccharides) (Courty et al., 2010). The role of fungi in weathering and in biogeochemical processes has received attention recently, in particular ectomycorrhizal fungi (Landeweert et al., 2001; Hoffland et al., 2004; Taylor et al., 2009) since these fungi, together with their connection to plants, represent an overall larger sink of nutrients and are potentially supplied with a large amount of photosynthetically fixed carbon (Rosling et al., 2009).

1.3.1 The role of ectomycorrhizal fungi in weathering

Weathering by ECM fungi was reviewed by Landeweert et al. (2001), Gadd (2007) and van Schöll et al. (2008). Rosling et al. (2003) found that the majority of ectomycorrhizal root tips may occur in deeper mineral horizons in a podzol soil rather than in the upper organic-rich horizons. Lindahl et al. (2007) also noted that mycorrhizal fungi dominate the total fungal community in mineral soil, suggesting that this is an important growth substrate for ectomycorrhizal fungi (Rosling et al., 2009). However, ECM species differ in their weathering capacity (van Schöll et al., 2006), through substrate acidification, organic ligand exudation, mineral type and different horizon colonization patterns (Dickie et al., 2002; Rosling et al., 2003; Casarin et al., 2004; Rosling et al., 2004). They have the ability to modify their chemical environment through local acidification around the hyphae and by exuding metal-complexing weathering agents such as organic acids (Rosling, 2009) and siderophores (Winkelmann, 2007).

Taylor et al. (2009) discussed different mechanisms by which ECM fungi contribute to mineral weathering. Through their mycelial interactions with mineral grains, secretion of low molecular weight organic compounds, uptake of cations and secretion of protons (direct mechanisms), and through their high rates of respiration, their promotion of plant growth, associated transpiration and litter production and their effects on litter decomposition rates and base depletion by leaching and soil organic matter accumulation (the last three considered indirect mechanisms). Organic acids of low molecular weight (LMWOAs) are considered the main agents of mineral dissolution, because of their dual acidifying and complexing properties (Barker et al., 1998), being oxalate one of the most widespread and abundant organic acids in forest soils (Jones et al., 2003). It has been extensively tested in weathering experiments where it has been shown to increase the dissolution of apatite (Wallander, 2000a; Wallander et al., 2003), biotite (Wallander, 2000b), phlogopite (Paris et al., 1996) and microcline (Wallander and Wickman, 1999). Other organic anions are also secreted by ECM fungi, such as citrate, formate, malate, malonate or succinate (Courty et al., 2010).

2 OBJECTIVES

N fertilization is a common practice to increase yield of commercial timber and biofuel production in intensively managed forests. It is now well established that an increase in N present in forest ecosystems can affect the composition and activity of the soil microbial community. However, little is known about the effect of N on the biogeochemical weathering processes that lead to mobilization of mineral nutrients essential for ecosystem functions. In the present work, the influence of N on fungal mediated-weathering of minerals in a boreal forest soil was evaluated. The main hypothesis tested was that N application would decrease the relative abundance and species diversity of fungi with the ability to colonise primary minerals and mobilize nutrients. For this purpose a laboratory microcosm experiment was conducted in pots using a forest soil, collected from the mor layer soil covering granite bedrock outcrops, amended with N and primary minerals. This experiment aimed at:

- Examine the changes in the fungal community composition, in particular the basidiomycete communities present in the forest soil with the addition of N and different primary minerals.

- Examine the changes in the availability and uptake of nutrients by plants in response to the addition of N and different primary minerals.

This experiment was part of a larger study and therefore the results included in this thesis are still preliminary. Nevertheless, it was already possible to draw some general conclusions about this topic.

3 METHODS

3.1 SITE AND SOIL SAMPLING

The forest soil used in this study was collected during February 2009 from the mor layer covering of granitic bedrock outcrops from a mixed forest dominated by Scots pine (*Pinus sylvestris* L.), Norway spruce (*Picea abies* L.), birch (*Betula spp*.), aspen (*Populus tremula* L.), and other less abundant deciduous trees, at Lunsen near Uppsala, Sweden (59° 45' N, 17° 45' E). Lunsen is a 13 km^2 nature reserve protected according to the EU–network Natura 2000. The climate is characterized by an annual mean temperature of 5.6 ºC and an annual mean precipitation of 544 mm (Alexandersson and Karlström, 2001).

Four sampling sites were chosen randomly within the area but always in close proximity to trees. Samples were taken from mats with a great proliferation of mycelia, suggesting an active fungal colonization of the rock surface (figure 1). Soil samples were put in plastic bags and stored in darkness at 4ºC until use. Soil preparation involved removal of large root fragments and upper soil layer covered with mosses and ground vegetation, followed by sieving (5 mm mesh), homogenization and storage at 16-18ºC. Moisture content was adjusted to 70% prior to the beginning of the experiment, by regularly measuring the water content of the soil until the desired value was reached. The moisture content was expressed as a percentage of the soil dry weight after drying soil samples in an oven at 100 ºC for 24h and weighing.

Figure 1. Mor layer covering granite bedrock outcrops in a mixed pine-spruce-birch forest at Lunsen near Uppsala, Sweden. Extensive fungal mats can be observed at the mor layer-rock interface.

3.2 EXPERIMENTAL DESIGN

3.2.1 Plant material

Scots pine (*Pinus sylvestris* L.) seeds (supplied by Saleby, Sweden) were surface sterilized in 30% hydrogen peroxide (H_2O_2) and germinated on vermiculite substrate in a plant propagator kept in a growth chamber at approximately 300 μmol m^{-2} s^{-1} PAR (photosynthetically active radiation) and with a 18h/6h 18 ºC/15 ºC day/night cycle for 40 days. They were watered with distilled water as needed.

3.2.2 Mineral preparation

The rock minerals used were quartz, apatite (both originated from Minas Gerais, Brazil and purchased from Krantz GmbH, Bonn, Germany), and biotite (originated from Kragerø, Norway and purchased from Krantz GmbH, Bonn, Germany). All the minerals were ground to a 250-400 µm particle size. The ground minerals were rinsed with distilled water for several hours until the pH was stable, to remove labile nutrients and the finest materials. After that, the minerals were left to dry in an oven at 80 ºC for 24h. The elemental composition of the minerals is given in table 1.

Table 1. Concentrations (mg g^{-1}) of several elements in quartz, apatite and biotite used in the present experiment.

Minerals	Elemental concentration (mg g^{-1})									
	Ca	K	Mg	P	S	Na	Mn	Al	Fe	Zn
Quartz	0.097	0.091	0.090	0.013	0.002	0.012	0.018	0.106	3.565	0.001
Apatite	318	0.384	0.047	191.5	4.415	0.755	0.157	0.615	1.468	0.054
Biotite	4.640	71.2	56.5	0.383	0.256	0.609	4.85	26.45	133.7	0.572

3.2.3 Microcosm

The laboratory microcosm experiment included different treatments in which the forest soil was amended with quartz, apatite or biotite (3% w/w), each with two levels of a slow release N fertilizer, methylene urea (39% N, Kemira, Finland) (1.6 g Kg^{-1} low N; 3.2 g Kg^{-1} high N) and a control without N. Other control treatments were also included, consisting of soil without minerals, and amended with the two levels of the slow release

N fertilizer or without N. Three replicates were set up for each treatment in a total of 36 microcosms for each sampling date.

Plastic pots (6 x 6 x 7 cm) were filled with homogenized mixtures using 83.5 g of soil, 0.75 g of each mineral and the adequate amount of methylene urea, as required for each treatment. The two rates of N used are similar to those applied in Swedish forests (about 200 kg ha^{-1} and 400 kg ha^{-1}, respectively). One pine seedling was then transplanted to each pot and the moisture content of the mixtures adjusted initially to about 75% and later to between 60-65%, using distilled water. These microcosms were kept in a growth chamber under the previously described conditions (figure 2). Germinating weeds and mosses were removed upon detection. All pots were randomised after each watering event to reduce potential position effects within the growth chamber.

An additional set of all treatments was kept in growth, consisting in the final sampling date of this experiment. However, it was not included in the results and discussion since this data was not available at the time. Still, in the following weeks after the 84 day harvest, it was possible to observe the degree of mycorrhiza present at the moment.

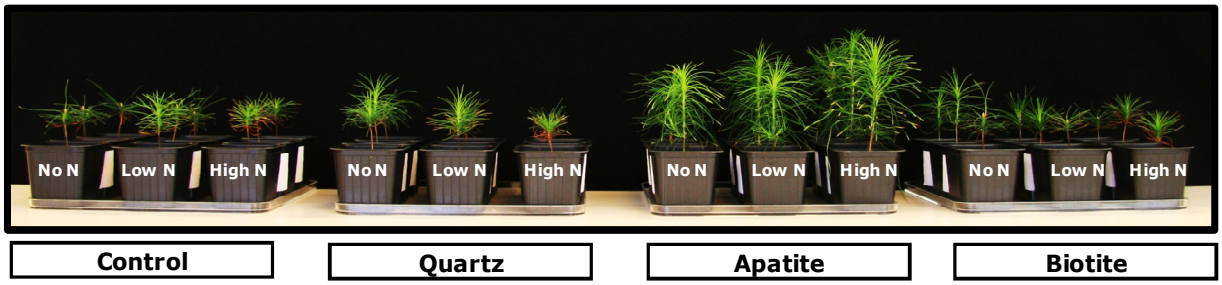


Figure 2. General view of microcosm experiment at day 84. Pine seedlings (*Pinus sylvestris* L.) were grown in microcosms with the mor layer amended with primary minerals (quartz, apatite, biotite or no mineral) and two concentrations of a slow release N fertilizer or without N.

3.3 HARVESTING PROCEDURE

Triplicates of each treatment were harvested destructively 0, 42 and 84 days after the beginning of the experiment. The replicates from each treatment were randomly selected and the seedlings removed. The top surface of the soil was discarded, and the remained soil was mixed in order to have a homogenised sample. Lastly, 10 g of soil were collected for molecular analysis and 60 g for chemical analysis. At day 84, the soil that adhered to the roots after gentle shaking was considered rhizosphere soil and sampled separately

from the remaining for molecular analysis. For each seedling, the root system was separated from the shoot, gently washed with distilled water to remove remaining soil particles and avoid mycorrhizal loss, and the approximate length was measured. All the samples were stored at -20 ºC until analysed.

3.4 PLANT ANALYSIS

For biomass determination shoots were oven-dried at 80ºC for 24h and weighed. Shoots were ground using a Mixer Mill MM 2 (Retsch, Düsseldorf, Germany) and for the 84 day harvest Ca, K, Mg and P analysis was performed by Agrilab AB (Uppsala, Sweden) using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) and, the Kjedahl method for N analysis.

3.5 SOIL ANALYSIS

Soils were centrifuged for 30 min at 3000 g to collect field-moist soil solutions, which were passed through a 0.45 µm filter and immediately frozen at -20 ºC until further analysis. The soil solution is considered here as the liquid phase of the soil. The pH (H_2O) was measured in previously collected soil solution using a pH meter (pHMeterS20, Mettler Toledo). Soil solution was sent to Agrilab AB (Uppsala, Sweden) for elemental analysis of Ca, K, Mg, P, Al and Fe by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES). The NH_4-N, NO_2/NO_3-N concentrations were determined with a Flow Injection Analyzer (FIA).

3.6 FUNGAL COMMUNITY ANALYSIS

For each sampling date, molecular profiling of the general fungal community and the basidiomycete community was determined by PCR - denaturing gradient gel electrophoresis (PCR-DGGE). The PCR-DGGE protocol generally consists of five major steps: sample collection, nucleic acid extraction, PCR amplification of the target genes, separation of PCR amplicons by DGGE, visualization of profiles and data analysis (figure 3).

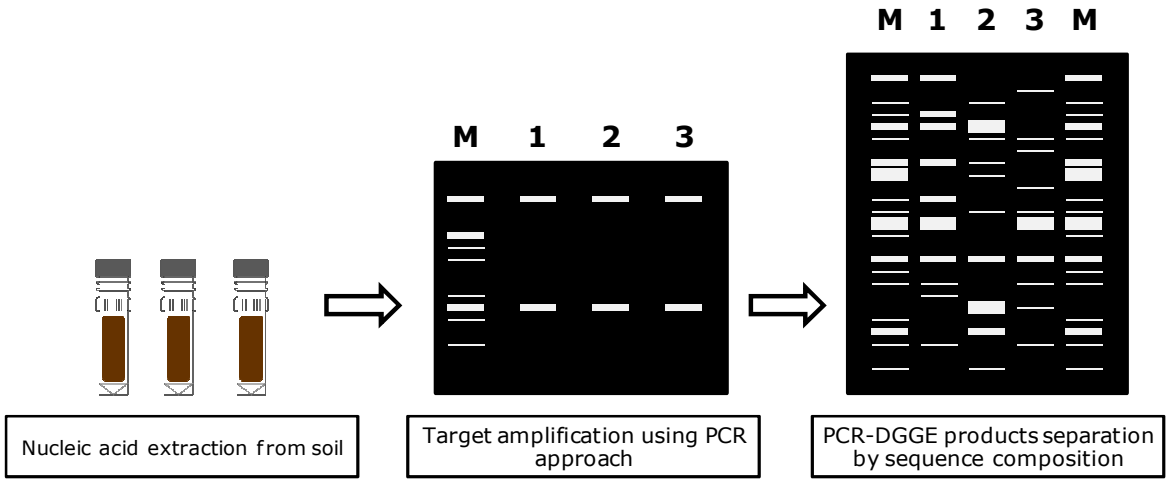


Figure 3. Flow diagram of the main steps for microbial community analysis using PCR-DGGE.

DNA was directly extracted from the soil samples and ITS amplified using a nested PCR approach for general fungal community and basidiomycete community. A nested PCR is a specific PCR amplification that uses two sets of primers, instead of one, to amplify a fragment. The second pair of primers is used in a secondary PCR reaction to bind inside the first PCR product fragment to allow amplification of a much smaller DNA fragment. The ITS region lies between the small subunit (SSU) and the large subunit (LSU) ribosomal RNA (rRNA) genes and contains 3 sub-regions, two non-coding spacer regions (ITS1 and ITS2) separated by the 5.8S rRNA gene. In fungi, this region is about 650-900 bp in size, including the 5.8S gene (figure 4) (Horton and Bruns, 2001; Nilsson et al., 2009). The ITS region has been the primary target in molecular identification of fungi in fungal community studies, mainly because the spacers ITS1 and ITS2 show a great rate of evolution, are typically species-specific and the large copy number of rRNA genes per cell makes the region easy to amplify, even from substrates where the initial amount of DNA is low, such as in soil samples (Gardes and Bruns, 1993; Nilsson et al., 2009).

Denaturing gradient gel electrophoresis (DGGE) was used to separate PCR-amplified DNA fragments in polyacrylamide gels containing a linearly increasing gradient of denaturants. DNA fragments of the same length but with different sequences can be separated (Muyzer et al., 1993; Muyzer and Smalla, 1998). The separation exploits the distinct mobility of double-stranded DNA (dsDNA) and partially denatured DNA in a polyacrylamide gel containing a linear gradient of DNA denaturants (a mixture of urea and formamide) that "melt" the dsDNA (Green et al., 2009). As dsDNA is denatured, the mobility is retarded (Muyzer et al., 1993). The denaturation of dsDNA or the so called *melting*, is based on the *melting domains*, which are characterized by base pair stretches

with identical denaturing temperature, since adenine-thymine bonding is maintained by two hydrogen bonds and guanine-cytosine bonding is maintained by three hydrogen bonds (Crick and Watson, 1954). Once the lowest temperature melting domain is reached, the partially melted DNA molecule stops migrating (Muyzer and Smalla, 1998). Sequence variation within such domains causes the melting temperatures to differ, and molecules with different sequences will stop migrating at different positions in the gel (Muyzer and Smalla, 1998). Full melting or strand separation is prevented by the presence of a high temperature melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC-rich sequence (GC clamp), previously added to the 5´-end of one of the PCR primers (Sheffield et al., 1989).

To obtain the best separation of the different DNA fragments, it is necessary to optimise the gradient and the duration of the electrophoresis prior to DGGE analysis. Afterwards DNA bands in DGGE profiles can be visualized using ethidium bromide, SYBR Green, SYBR Gold or silver staining.

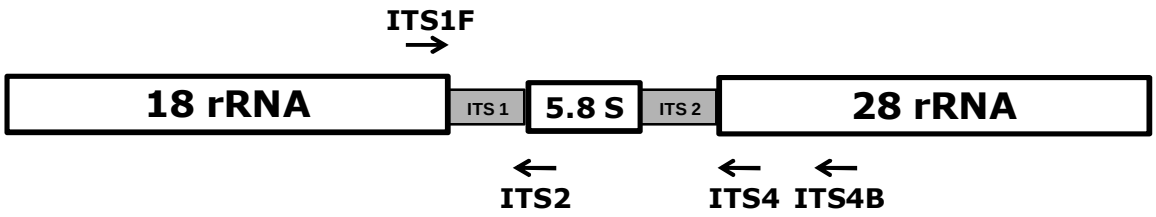


Figure 4. Schematic representation of ribosomal RNA genes with annealing sites of primers (modified from Gardes and Bruns, 1993).

3.6.1 DNA extraction

Nucleic acids were extracted from 0.5 g soil using the method developed by Griffiths et al. (2000). Briefly, cells were lysed for two cycles of 30 s with Precellys 24 (Bertin Tecnologies, Montigny-le-Bretonneux, France) at a speed setting of 4 ms^{-1} in 2.0 mL Lysing matrix B tubes (MP Biomedicals, Illkirch, France) with silica spheres containing 0.5 mL of hexadecyltrimethylammonium bromide (CTAB) extraction buffer, 0.25 mL of phenol-chloroform–isoamyl alcohol (25:24:1 v/v, pH 8.0) and 0.25 mL of chloroform-isoamyl alcohol (24:1). The aqueous phase containing the nucleic acids was separated by centrifugation (16000 g) for 10 min at 4 ºC. Phenol was removed by adding 0.5 mL of chloroform-isoamyl alcohol (24:1) followed by repeated centrifugation (16000 g) for 10 min at 4 ºC. Further precipitation of nucleic acids from the extracted aqueous layer was

obtained by adding two volumes of 30% (w/v) polyethylene glycol 6000 – 1.6 M NaCl for 1 h on ice followed by centrifugation (16000 g) at 4 ºC for 30 min. Pelleted nucleic acids were then washed in ice-cold 70% (v/v) ethanol, air-dried and resuspended in 30 µL of sterile water.

3.6.2 PCR amplification

The primary PCR used a general fungal primer pair, ITS1F and ITS4, and a basidiomycete specific primer pair, ITS1F and ITS4B. ITS1F and ITS4 are considered a fungal selective ITS primer (White et al., 1990) and ITS4B is intended to be specific for basidiomycetes (Gardes and Bruns, 1993). A secondary PCR with ITS1F-GC and ITS2 (White et al., 1990) primers was used to generate products suitable for community fingerprinting using denaturing gradient gel electrophoresis (DGGE). Table 2 summarizes the primer sequences and figure 4 the annealing sites.

All PCRs were carried out in 50 µL reaction volumes containing 2 µL of template DNA from 1:50 dilutions, 5 µL of 10 x NH_4 PCR buffer (Bioline, London, UK), 1.5 µL of 50 mM $MgCl_2$ solution (Bioline, London, UK), 1 µL of 20 mg (1 mL) bovine serum albumin (Roche Applied Science, Indianapolis, USA) for primary PCR only, 1 µL of each primer (10 µM), 1 µL of 12.5 mM dNTPs mix (Larova GmbH, Teltow, Germany) and 0.2 U of Biotaq™ DNA polymerase (Bioline, London, UK).

All amplifications were carried out on a Veriti 96 well thermal cycler (Applied Biosystems). The thermocycling conditions for both primer-sets were preceded by an initial denaturation step (94 ºC for 5 min) and followed by a final elongation step phase (72 ºC for 10 min). For each cycle of PCR, denaturation was at 94 ºC for 30 s, annealing at 55 ºC for 30 s and elongation at 72 ºC for 30 s. In addition to the target soil DNA, a 'negative' (without DNA) control sample was included in every PCR run. PCR products were examined by standard 1% (w/v) agarose 1 x Tris - acetate – EDTA (TAE) buffer gel electrophoresis (Sambrook et al., 1989) with 10 000 x in DMSO gelgreen™ (Biotium, Hayward, CA) staining, to confirm product integrity.

Table 2. Summary of nested PCR primers used for assessing fungal diversity in soil DNA extracts.

PCR primer	Primer Sequence (5′ – 3′)	References
ITS1F	CTTGGTCATTTAGAGGAAGTAA	White et al., 1990; Gardes and Bruns, 1993
ITS4	TCCTCCGCTTATTGATATGC	
ITS4B	CAGGAGACTTGTACACGGTCCAG	
ITS1f-GC	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCTTGGTCATTTAGAGGAAGTAA	
ITS2	GCTGCGTTCTTCATCGATGC	

3.6.3 DGGE analysis

Denaturing gradient gel electrophoresis analysis (DGGE) of PCR products was carried out using the D-Code Universal Mutation Detection System (Bio-Rad, Hemel Hempstead, UK), using the method of Muyzer et al. (1993). The procedure involved solution preparation, apparatus cleaning and assembly, gradient casting and polymerization, sample loading, electrophoresis, staining, and gel imaging. Briefly, polyacrylamide gels (40% Acrylogel 2.6 solution; BDH Laboratory Supplies, Poole, UK) containing gradients of 20–50% denaturant were prepared using a gradient maker (Fisher Scientific, Loughborough, UK) coupled to a peristaltic pump (5 ml min^{-1}). The denaturing solution strength of 100% was defined as 7 M urea and 40% formamide. A non-denaturing stock solution (0% polyacrylamide) was prepared to serve as foundation (plug) and top-up (stacker) before and after the denaturing layer was poured, respectively. Gelbond PAG film (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was hydrophobically bonded to the small glass plate and gels were poured unto the hydrophilic side, in order to facilitate handling of gels during the staining procedures. All solutions were polymerized with 10% ammonium persulfate (APS) solution and TMED (N,N,N',N'-Tetramethylethylenediamine). Fifteen µL of each PCR product was loaded together with 5 µL of loading buffer to help the visualization of wells. Gels were run for 16 h at 75 V in 1 x Tris-acetate EDTA (TAE) buffer at a constant temperature of 60 ºC. To enable comparison between gels, all gels included three marker lanes, consisting of a PCR product from one of the samples from day 0, randomly picked.

Following electrophoresis, the gels were silver-stained according to Mahmood et al. (2006) and scanned. Silver-staining of DGGE gels involved incubation of the gel in a fixing solution (10% ethanol + 0.5% acetic acid) for 15 min followed by incubation in silver nitrate solution (0.2% silver nitrate dissolved in the fixing solution) for 15 min, three washes in double-distilled water (ddH_2O) for 3 min and incubation with the developing solution (3% NaOH + 0.5% formaldehyde) for 10 min. The developing solution was removed and the gels washed with ddH_2O for 2 min, followed by fixation for

10 min and further rinsing with ddH_2O. The gels were then scanned (Epson Perfection V700 Photo, Seiko Epson Corporation, Japan) by using Epson Scan software.

3.6.4 Recovery and purification of DNA template from DGGE bands and sequencing

Eleven representative, prominent bands from basidiomycete profiles of rhizosphere soil samples at the 84 day harvest were excised and sequenced. The chosen bands were found to respond to the treatments tested. To facilitate comparisons between banding profiles from the three sampling dates (0, 42 and 84 days after the beginning of the experiment), selected samples were run on a single DGGE gel and the positions of the bands of interest were analysed in relation to the DGGE control markers (with known mobility in the gel). The bands that migrated to a similar position on the gel were presumed to have similar sequences and therefore only representative bands (with identical migratory positions) were excised for subsequent DNA sequence analysis. DGGE gels were run as described above, except that they were stained with a 1:10 000 dilution of SYBR Gold nucleic acid stain (Invitrogen, Oregon, USA) (Tuma et al., 1999). The excision was done with a sterile razor while the gels were illuminated on a Dark Reader (Clare Chemical Research, Detroit, USA) and the DNA was eluted by incubation of the crushed band in 50 µL sterile ddH_2O at 65 ºC for 30 min, followed by centrifugation at 5000 g for 3 min. The eluted DNA (1 µL) was used as template for PCR amplification with primers ITS1F-GC-ITS2, under the conditions described above, and the resultant amplified products were analysed on an agarose gel. The purity of amplification products was assessed by DGGE analysis and, since products yielded several bands, in addition to the band of interest, further rounds of band excision, PCR amplification and DGGE analysis were performed until a suitable purity was achieved.

The purified PCR products were sequenced with the primers ITS1F and ITS2 by Macrogen Europe (Amsterdam, The Netherlands). The BLASTN search tool (Altschul et al., 1990) was used to find sequence homology and to determine the most similar sequences in the GenBank database.

3.7 STATISTICAL ANALYSIS

The experiment consisted of a factorial design of 4 mineral treatments (three primary minerals and a non-mineral control), 3 N levels (low N, high N and a non N control) and 3 replicates for each treatment. Plant growth parameters, soil pH and soil and shoot elemental parameters were analysed using analysis of variance (ANOVA). The effect of N and minerals on all the parameters analysed were tested in a two-way ANOVA. The data from the apatite and biotite treatments was also submitted to a one-way ANOVA to investigate the effects of N on the elemental concentrations of shoots and soil solution.

Normality was tested with a Shapiro-Wilks test and by inspection of residuals, and variance homogeneity by Levene's test. In order to comply with analysis of variance (ANOVA) assumptions for homogeneity (Levene's test), potassium concentration in shoots was exponential transformed before analysis.

The Tukey's honestly significant difference (HSD) method was used for *post hoc* comparisons with a 0.05 grouping baseline. Pearson's linear correlation coefficients were calculated to detect relationship between parameters. All statistical analyses were performed with the STATISTICA 7.0 software (StatSoft Inc., Tulsa, OK, USA).

4. RESULTS AND DISCUSSION

The influence of N application on the fungal communities involved in the mobilization of nutrients from primary minerals was analysed by evaluating changes in PCR-DGGE banding profiles. Plant growth parameters, soil solution and shoot elemental concentrations were then analysed in order to make a primary assessment of the impact of N on the mobilization of nutrients.

4.1 EFFECT OF NITROGEN ON FUNGAL COMMUNITY STRUCTURE

General fungal community profiles were first generated because of the overall significant role of fungi in mineral dissolution and secondary mineral formation (Whitelaw, 2000; Gadd, 2004; Rosling et al., 2007). Very rich communities were obtained but only reduced temporal and treatment-specific differences could be detected with the naked eye (appendix A).

Mycorrhizal fungi are one of the most ecologically important groups of soil fungi in terms of mineral transformations and redistribution of inorganic nutrients (Paris et al., 1995; Jongmans et al., 1997; Gadd, 2007). The organic mor layers provide a unique habitat for microbial life and extensive fungal mats are often observed at the mor layer-rock interfaces. Certain species of ectomycorrhizal fungi form these mat-like structures (Landerweert et al., 2001). Ectomycorrhizal associations are formed by members of the Basidiomycota and to a lesser extent, Ascomycota (Smith and Read, 2008). Therefore, ECM fungi are not a monophyletic group, and ECM-specific primers do not exist (Landeweert et al., 2003b). Since a majority of ECM fungi are basidiomycetes (Gardes and Bruns, 1993), PCR-DGGE fingerprints of basidiomycete communities were produced in order to detect changes in community composition. DGGE fingerprinting provides a powerful tool to assess the microbial diversity in environmental samples (Fromin et al., 2002).

4.1.1 Changes in basidiomycete community structure

As expected, at the beginning of the experiment samples were highly homogeneous, as confirmed by similar DGGE profiles (figure 5 A). Treatment-associated changes became more pronounced at day 84 although more variation between replicates was also detected. Nevertheless, temporal- and treatment-related differences indicated by PCR-DGGE provided an indication of community changes. Eleven bands were selected in

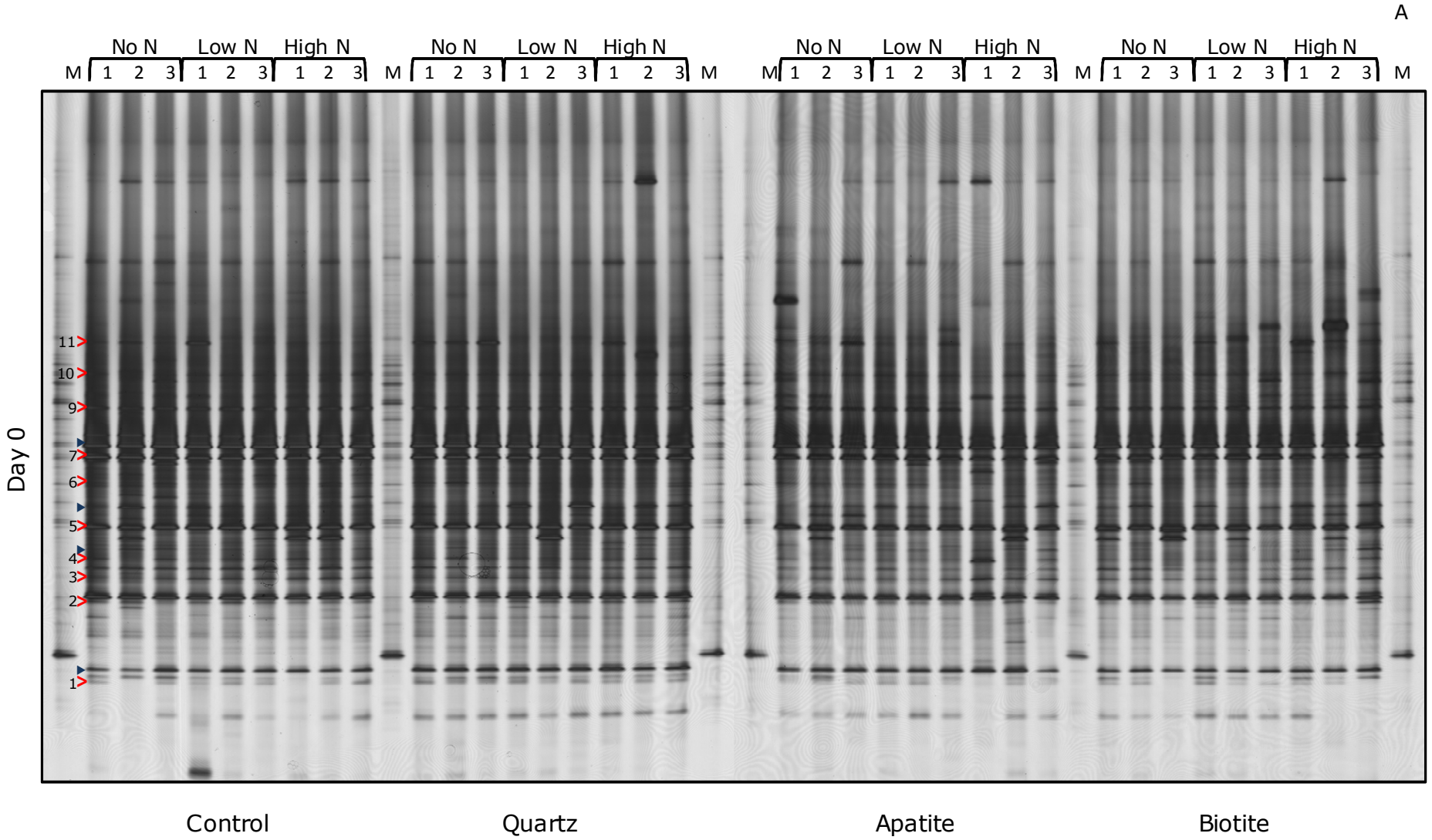
DGGE profiles (figure 5 A, B, C, D), excised and sequenced (table 3). Community analyses using sequence similarities can provide insights into the taxonomic level at which microbes respond to N. All of the sequenced bands were from ectomycorrhizal fungi (table 3) belonging to the families Russulaceae and Thelephoraceae (except band B2 and band B7). It has been shown that members of Russulaceae and Thelephoraceae are among the most abundant and frequent taxa on ECM roots in conifer communities (Horton and Bruns, 2001).

Four extra bands that followed similar patterns were excised and sequenced, but no conclusive results were obtained due to low similarity or lack of similarity in the GenBank data base. Therefore, they were not included in the sequencing results but their position was identified in the gels for corroboration of visual analysis (figure 5 A, B, C, D).

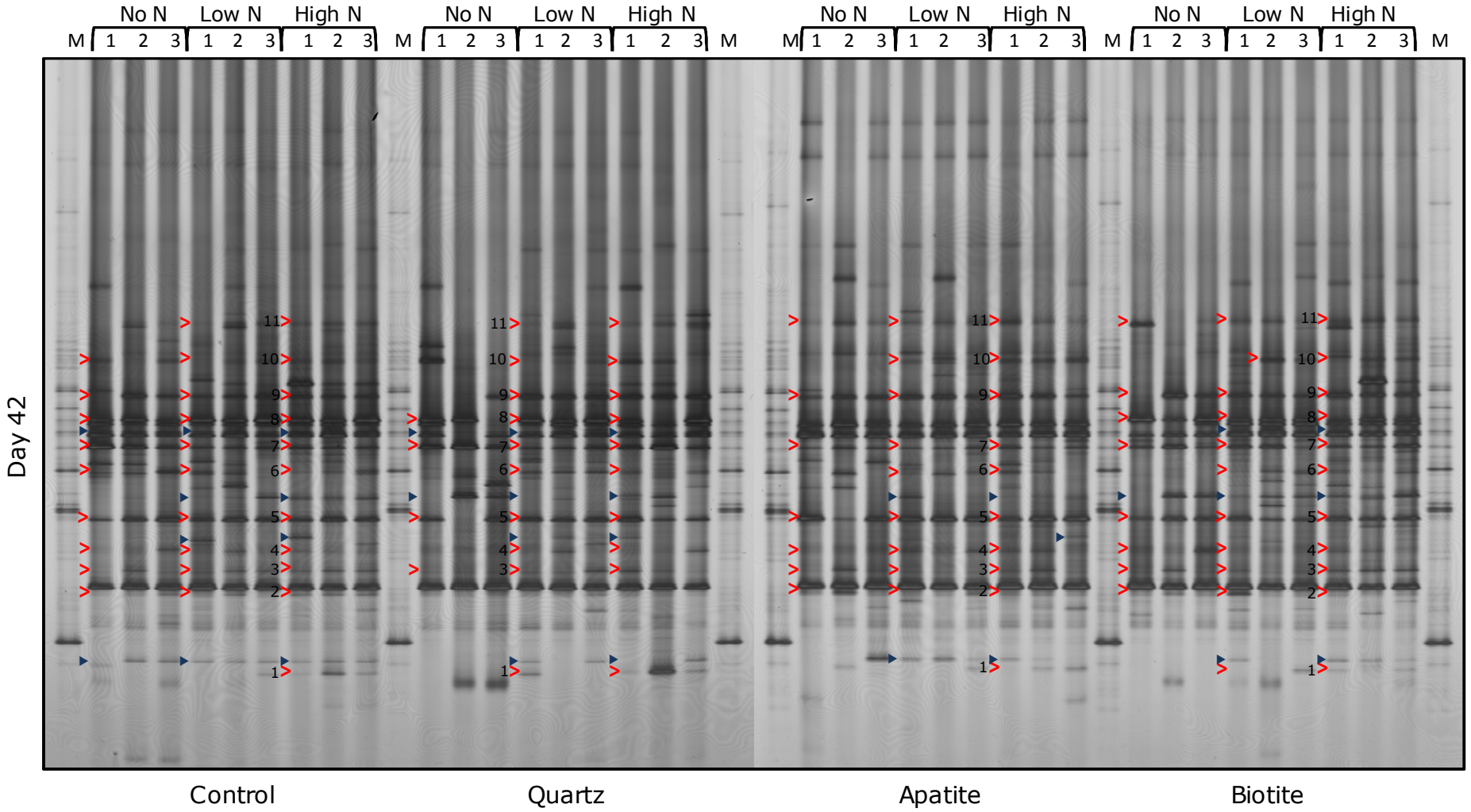
Table 3. Closest matches between DNA sequences of bands excised from denaturing gradient gel electrophoresis (DGGE) gels and sequences from the GenBank databases obtained using the BLASTN search tool.

Band no.	Closest relative in GenBank (accession number)	Percentage similarity *
B1	Uncultured Tomentella isolate 7640.2 (EU668250.1)	98
	Uncultured ectomycorrhiza (Tomentella) isolate FRA4 (AY748877.1)	98
B2	Uncultured fungus clone S19 (FJ820507.1)	97
	Uncultured ectomycorrhiza (Basidiomycota) (AY641458.1)	91
B3	*Lactarius rufus* voucher K80S07 (GQ267478.1)	99
	Lactarius rufus isolate rufus008 (EF685089.1)	99
B4	*Russula paludosa* (AJ971400.1)	94
	Russula paludosa (AJ971402.1)	94
B5	Uncultured ectomycorrhiza (Russula) clone ECUBC39 (EU057103.2)	99
	Russula vinosa (AF418638.1)	99
B6	*Lactarius sp.* 'Bremen H' (AF438577.1)	99
	Lactarius helvus (AY606946.1)	98
B7	*Piloderma fallax* isolate UP113 (DQ179125.1)	98
	Piloderma fallax isolate HJA2138 (AY534198.1)	98
B8	*Pseudotomentella humicola* ITS1 (AM490945.1)	100
	Pseudotomentella humicola ITS1 (AM490946.1)	98
B9	Uncultured ectomycorrhizal fungus (AJ633582.1)	97
	Uncultured ectomycorrhiza (Thelephoraceae) (EF077521.1)	100
B10	Uncultured ectomycorrhizal fungus (AJ633582.1)	98
	Uncultured Tomentella isolate VII.cm118.ps (EU668280.1)	97
B11	Uncultured Pseudotomentella (FN393141.1)	99
	Pseudotomentella tristis (AM087266.1)	99

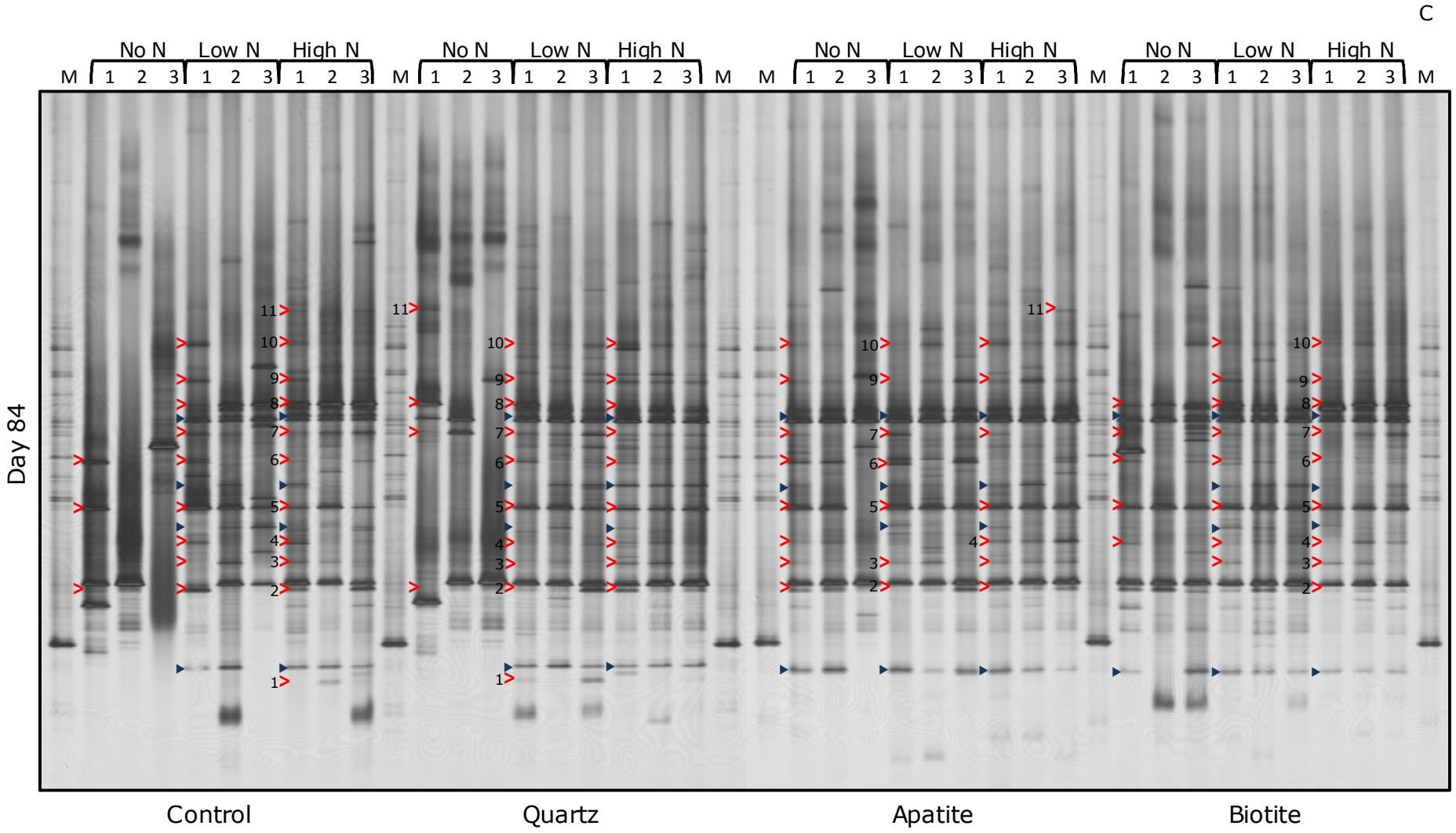
Bands excised corresponded to bands that showed some response to N addition.
* Percentage similarity between the sequence of a band excised from DGGE gel and the closest match in GenBank.


A
No N
Low N
High N
M 1 2 3 1 2 3 1 2 3 M
11
10
9
7
6
5
4
3
2
1
Day 0
Control
Quartz
Apatite
Biotite

B

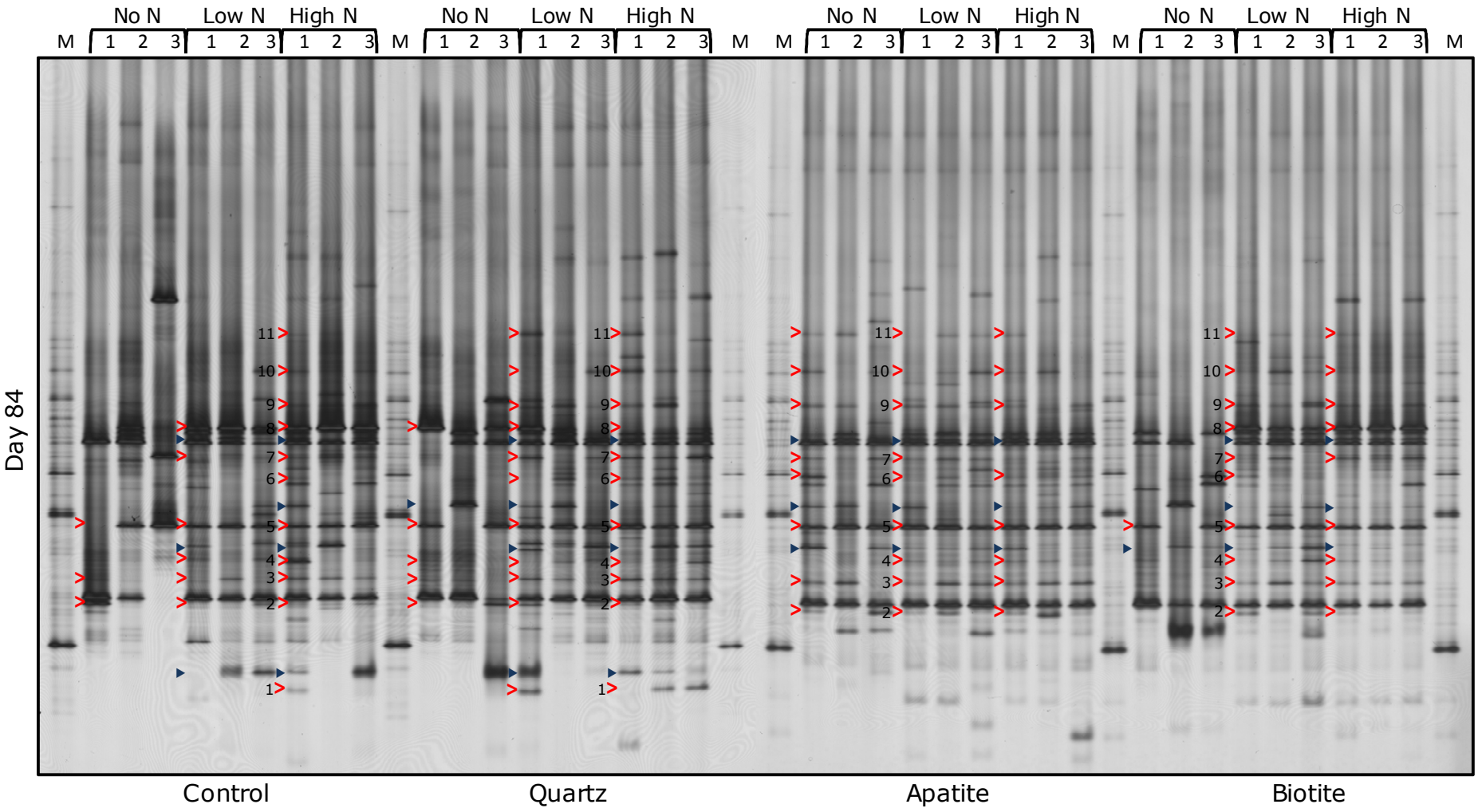

No N
Low N
High N
M 1 2 3 1 2 3 1 2 3 M
Day 42
Control
Quartz
Apatite
Biotite

Bulk Soil



Rhizosphere Soil

D



← **Figure 5.** Denaturing gradient gel electrophoresis (DGGE) profiles of the Basidiomycete communities based on ITS regions from a natural forest soil amended with different N levels (No N, Low N, High N) and primary minerals (Control with no minerals added, Quartz, Apatite, Biotite). Lanes 1-3 in all treatments are samples from triplicate microcosms that were harvested destructively. Arrowheads indicate bands present in N treatments or with greater intensity in response to N treatments compared with controls. Bands 1-11 were excised and sequenced (for identities see table 3). Markers in lane M consisted of one of the samples from day 0, randomly picked. A) DGGE profile from soil at day 0, B) DGGE profile from soil at day 42, C) DGGE profile from bulk soil at day 84, D) DGGE profile from rhizosphere soil at day 84.

The effect of N on fungal community structure was detected visually. The basidiomycete communities started to change from day 0 and by day 84 fewer bands were present, as well as a diverse banding pattern, suggesting that changes in the community were still occurring at the time of the harvest. Most of the bands sequenced were influenced by N addition since they were absent in the NN treatment or in relatively lower intensity at day 84 compared to the LN and HN treatments (bands B1, B3-B4, B6-B11), both in bulk soil and rhizosphere soil (figures 5 C, D). Bands marked in the gels but with no sequence results also showed the same effect of N.

Rhizosphere soil demonstrated higher banding richness compared to bulk soil. Rhizosphere soil is defined as the soil adjacent to the root system and that is influenced by the root exudates (Kent and Triplett, 2002; Mukerji et al., 2006). It is a niche where accumulation of complex microbial communities around the root zone is supported by different compounds exuded by roots and also capable of a selective effect on the microorganisms within the root zone (Mukerji et al., 2006). Band B5 showed a high intensity through time in all treatments, except in bulk soil at day 84 where it almost disappeared in the CNN and QNN treatments compared to the rhizosphere soil. Also, band B11 was almost absent in bulk soil. At day 42, when rhizosphere soil was not separated, bands with higher intensity were present as well as less variation between N treatments (figure 5 B). It is assumed that band intensity is directly related to the relative abundance of the corresponding phylotype (Murray et al., 1996; Fromin et al., 2002).

As time passed, most bands decreased in relative intensity but bands B1, B7 and B9 in particular diminished substantially. Bright yellow *Piloderma fallax* (band B7), a species earlier referred to as *P. croceum*, is known to be a mat-forming ectomycorrhizal fungus (Kranabetter et al., 2009; Kluber, 2010) and, together with the rest of the genus *Piloderma* has an ecological importance in many boreal forest ecosystems (Erland &

Taylor, 1999). *Piloderma* mats are one of the most visually striking ECM mats due to their tendency to heavily colonize the soil with thick, cord-like rhizomorphs (Kluber, 2010). When collecting samples in the Lunsen forest, fungal mats demonstrated a large proportion of bright yellow mycelium (figure 6). However, at the 84 day harvest, the proportion of bright yellow mycelium had greatly decreased. The reduction in band B7 intensity throughout time, together with the apparent lack of mycelium at day 84 supports the decline in relative abundance of *Piloderma fallax*.

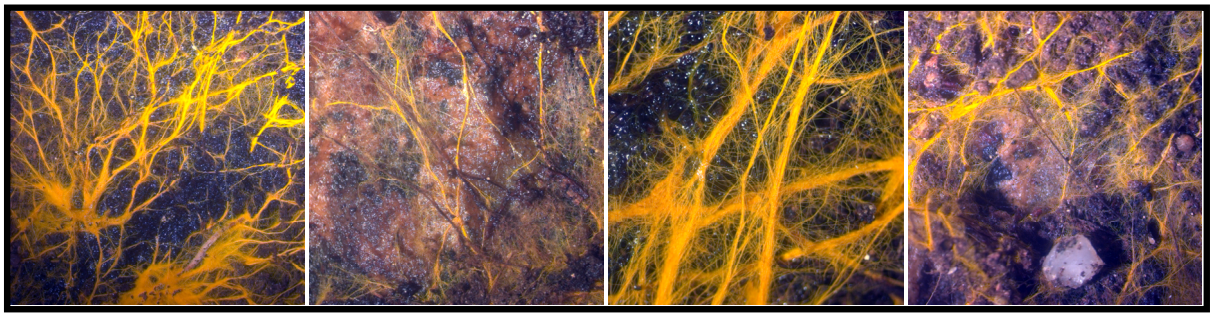


Figure 6. Fungal mycelium present in the rock surface from granite bedrock outcrops in contact with mor layer soil collected from mixed pine-spruce-birch forest at Lunsen near Uppsala, Sweden.

It was not possible to make conclusive statements about the effect of minerals on fungal community structure because of the complexity of the DGGE profiles and variability between treatments. Computer assisted characterization of the banding patterns and subsequent analysis of the data using a multivariate statistical approach will provide refined results based on the comparison of both the position and the relative intensity of different bands within gels (Fromin et al., 2002). Nevertheless, mineral treatments seem to have affected band B1 since at day 42 it was only present in the N treatments but at day 84 disappeared completely from apatite and biotite treatments, both in bulk soil and rhizosphere soil. One of the bands marked with no sequence results (the first from the bottom) also disappeared during apatite and biotite treatment at day 84 in the rhizosphere soil. Band B8 was absent in the apatite treatment (figure 5 B, C, D). However it should be kept in mind that the interpretation of the disappearance of bands as a complete absence of that species in the community can be misleading. In fact, it may only reflect a change in the relative densities between populations within the community, where the increase in some populations forces other populations below the detection limit of DGGE (Nakatsu, 2007).

The apatite treatment had a different banding pattern compared to the rest of the treatments. NN treatments from control, quartz and biotite amendments showed

relatively lower band richness compared to the LN and HN treatments. Nitrogen addition seemed to have increased species richness and relative abundance in these treatments, in contrast to the initial hypothesis that basidiomycete fungi would decline in relative abundance and richness through time, in response to increasing N availability (Parrent et al. 2006; Taniguchi et al. 2007). This suggests a selection towards N tolerant species or alternatively, the basidiomycete community in the soil was relatively deficient in N and the addition improved fungal growth. However, most bands that were affected by N in control, quartz and biotite treatments did not show a clear effect of N under apatite treatment. In fact, most of them were also present in NN treatment in addition to LN and HN treatments, with variable intensities across replicates. It seems that apatite addition alone improved basidiomycete richness in the soil, independently of the N treatments (figure 7) and therefore, the enhanced band richness and abundance observed in the treatments with other minerals and N was not evident under apatite amendment.

Many soil fungi are able to solubilize P directly from minerals such as apatite (Rosling et al., 2007). There are indications that P uptake of host trees increases with increasing ectomycorrhizal diversity (Baxter and Dighton, 2001). It is therefore possible to hypothesize that increased availability of P in the soil due to apatite addition increased ectomycorrhizal richness or selected fungi with specific abilities to acquire P from apatite. *Piloderma* is known to influence nutrient uptake and mineral transformation in rock and soil systems (Jongmans et al., 1997; Arocena et al., 1999). Tuason and Arocena (2009) provided evidence that *Piloderma fallax* has an important role under limited P availability because of an increase in production and release of soluble oxalate. In the present study N addition enhanced P limitation in control, quartz and biotite treatments (see following chapters) and therefore it may have contributed to the higher abundance of the species under LN and HN treatments compared to the NN treatment. However, apart from *Piloderma fallax*, it was not possible to find direct evidence in the literature that the taxa identified in the PCR-DGGE profiles affected by N addition may have a direct role in the weathering of primary minerals. This is because most species-rich genera in ECM communities (such as *Cortinarius*, *Lactarius*, *Russula* or *Tomentella* sp.) are very difficult to isolate and culture *in vitro* (Courty et al., 2010) and several weathering studies have only been performed in laboratory conditions.

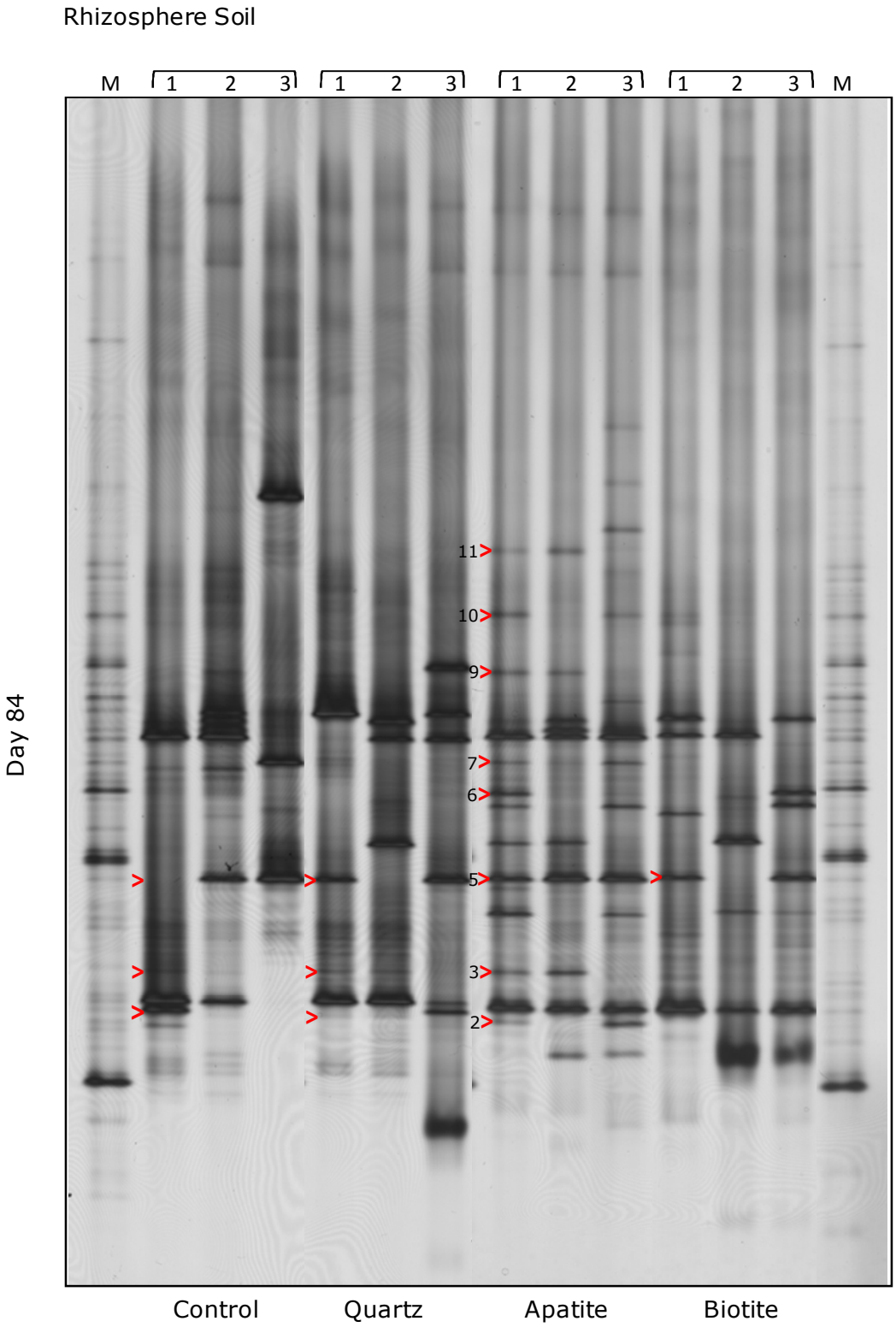


Figure 7. Denaturing gradient gel electrophoresis (DGGE) profile from rhizosphere soil at day 84 of the basidiomycete community based on ITS regions from a natural forest soil amended with primary minerals (Control with no minerals added, Quartz, Apatite, Biotite). Lanes 1-3 in all treatments are samples that were harvested destructively. Bands marked with numbers were excised and sequenced (for identities see table 3). Markers in lane M consisted in one of the samples from day 0, randomly picked.

4.1.2 Effect of nitrogen on basidiomycete communities

Ectomycorrhizal symbiosis is not only characterized by the presence of colonized root tips but also by the development of extramatrical mycelia radiating into the soil (Landeweert et al., 2003). The identification and quantification of mycelia in soil have only recently been studied by molecular methods based on the extraction of total soil DNA which facilitates identification of hyphae directly from the environment irrespective of morphology or growth stage. (Dickie et al., 2002; Guidot et al., 2002; Landeweert et al., 2003, 2003b). It is likely that the mycelial view of the ECM community will be different from both root and fruit-body views and, the relationship between mycelial investment and root dominance largely unknown (Horton and Bruns, 2001). A relatively small number of dominant fungal taxa can form most of the ECM root tips present in nature (Gardes and Bruns, 1996; Erland and Taylor, 2002) whereas the majority of species are only rarely encountered (Gehring et al., 1998; Grogan et al., 2000; Peter et al., 2001). Therefore, some caution is needed when analyzing PCR-DGGE banding profiles since they are based on total soil DNA, which makes it impossible to infer anything about ECM root colonization.

Elevated N levels can alter mycorrhizal symbioses (Avis et al., 2008), as it has been shown in conifer dominated ecosystems (Avis et al., 2003). Numerous studies of the effects of forest fertilization and N deposition on ECM fungi based on sporocarp abundance (Rühling and Tyler 1991; Wiklund et al. 1994; Brandrud 1995; Jonsson et al. 2000; Lilleskov et al., 2001; Peter et al., 2001; Avis et al., 2003) and mycorrhizal root tips (Kårén and Nylund, 1996; Fransson et al., 2000; Jonsson et al., 2000; Peter et al., 2001; Lilleskov et al., 2002; Avis et al., 2003; Frey et al., 2004; Berch et al., 2006; Avis et al., 2008; Wright et al., 2009) have reported changes in the ECM community structure, generally resulting in reduction of sporocarp production, abundance on root tips, reduced species richness and changed community composition. However, sporocarp communities are not directly representative of belowground communities (Dahlberg et al. 1996; Gardes and Bruns, 1996; Kårén and Nylund 1996) since many important ECM species do not form aboveground sporocarps or form inconspicuous reproductive structures (Taylor and Alexander, 1989; Dahlberg et al. 1996; Kårén and Nylund 1996). Changes in the availability of inorganic nutrients can also influence growth and production of extramatrical mycelium. Such availability is also influenced by the type of fertilization used (Fransson et al., 2000), and thus changes in the fungal community structure are dependent on the nutrient status of the soil and plants in addition to the type and amount of fertilizer applied within a given period of time.

In the present study, sequencing of DGGE bands provided evidence for selection of different ECM fungi in the LN and HN treatments. Bands B1 and bands B8-B11 belonged to the family Thelephoraceae, which produce inconspicuous sporocarps. Bands B1 and B10 were identified to the genus level, *Tomentella* and B8 and B11 to species level, *Pseudotomentella humicola* and *Pseudotomentella tristis*, respectively. Tomentelloid fungi appear to be relatively common ectomycorrhizal symbionts with a wide distribution in Swedish coniferous forests (Kõljalg et al., 2000) and the *Tomentella* genus has been reported to be a major component of the ECM community in several forests (Horton and Bruns, 1998; Taylor and Bruns, 1999; Kaldorf et al., 2004). Kõljalg et al. (2000) studied the presence and abundance of tomentelloid fungi in Swedish forests, demonstrating that *Pseudotomentella tristis* was one of the most commonly found fungi belonging to this order. Peter et al. (2001) reported that the proportion of species belonging to the Thelephoraceae on root-systems in a stand of subalpine spruce (*Picea abies*) increased at higher N levels. Fransson et al. (2000) and Taniguchi et al. (2007) also reported the increase of *Tomentella sp.* in ECM root tips due to N fertilization and deposition, respectively.

Many studies reported that *Russula sp.* appear to be sensitive to N (Ohenoja, 1988, 1989; Brandrud, 1995; Lilleskov et al., 2001; Peter et al., 2001; Lilleskov et al., 2002). However, Avis et al. (2003) and Avis and Charvat (2005) showed a greater abundance of *Russula sp.* in an oak savanna ecosystem and the former suggested that it may benefit from a high N supply when other factors caused by N addition such as soil acidification, cation loss or P limitation are not present. In the present experiment, that may not be the case as although N did not cause soil acidification, it might have enhanced P limitation and cation loss because fewer cations were mobilized into the soil solution (see following chapters). It was also suggested that the Russulaceae have special adaptations that allow host plants to benefit when N supply is high, such as improved P acquisition due to release of oxalates (Avis et al., 2003).

Lactarius sp., also belonging to the family Russulaceae, are usually considered to be nitrophilic (Brandrud, 1995; Lilleskov et al., 2002) since a general increase in abundance is often observed in association with increased N availability (Taylor et al., 2000; Lilleskov et al., 2002). In a forest fertilization experiment, Brandrud (1995) demonstrated that fruit-body production of *Lactarius rufus* (band B3) increased temporarily with N deposition, as it has been reported in other fertilization experiments (Ohenoja, 1988, 1989). Kårén (1997) also found an increase in the percentage of root tips colonized by *Lactarius rufus,* although fertilization resulted in a decrease in the fruiting of that species.

Piloderma spp. have been shown to decline in abundance along gradients of increasing N availability in the field (Lilleskov et al., 2002; Toljander et al., 2006) and after N fertilization (Kårén, 1997; Fransson et al., 2000). However, visual assessment of PCR-DGGE banding profiles showed that although *Piloderma fallax* decreased in relative abundance throughout time, a similar reduction did not occur after N addition, suggesting instead the opposite effect, i.e. a higher abundance under N treatments compared to treatments without N.

4.1.3 Conclusions

Caution is needed when interpreting these results, since most of the studies on effects of N on ECM fungi are so far based on root colonization and sporocarp production and do not take into account the active mycelium present in the soil. On the other hand, the total soil DNA approach applied in this experiment shows only that DNA sequences obtained belonged mainly to fungi that were abundant at the time of sampling and does not distinguish between mycelium present in the soil and root fungal colonization, which makes it difficult to establish a relationship between the effect of N on fungi colonizing roots and comparison between studies.

The colonized root tip is considered the functional symbiotic unit in ectomycorrhizal fungi, where carbon is transferred from the host tree to the fungus and nutrients obtained by the fungus transferred to the plant (Smith and Read, 2008). Carbon is thus considered the driver of ectomycorrhizal weathering activity (Rosling et al., 2009). However, it is not possible to link the identity of ECM fungi colonizing roots to the degree of root and mycelial soil colonization which together with the effect of the chemical activity of their mycelia, should be factors influencing the degree of weathering activity by ectomycorrhizal fungi (Wallander et al., 1997). Rosling et al. (2003) and Landeweert et al. (2003b) showed that although ectomycorrhizal species composition on root tips was generally similar to the species composition of extraradical mycelia, in several cases species that were abundant on root tips were not detected in soil extracts, and others that were abundant in soil extracts were rarely detected on roots. Several other studies demonstrated that ECM species dominant in one of the three communities, sporocarps, root tips, or hyphae, are often a small component of the other two communities (Horton and Bruns 2001; Smith et al., 2007; Porter et al., 2008). Hynes et al. (2010), showed that the hyphal community in a Californian *Quercus–Pinus* woodland was mostly dominated by taxa different from those characterizing either the sporocarp or ECM root communities. In conclusion, fungal biomass quantification and sequencing of fungal root tips can help to confirm the relative abundance of bands observed in the PCR-DGGE profiles and relate their identities to the dominant fungal taxa colonizing the host tree.

4.2 MOBILIZATION OF NUTRIENTS FROM PRIMARY MINERALS

An assessment of the weathering of primary minerals was performed on the basis of data currently available. This study differs from the majority of microcosm experiments because it used a forest soil, not an artificial substrate that poorly represents a natural environment. However, to examine weathering activity becomes more difficult because of the difficulty of quantifying all the factors involved.

4.2.1 Seedlings development

Shoot biomass was significantly affected by N, minerals and the N x minerals interaction, but root length was only significantly affected by N and minerals (table 4). In the present study, addition of apatite enhanced shoot growth and root length of pine seedlings at day 84 compared with biotite, which did not increase growth. Apatite is the primary mineral source of P in the soils (Rogers et al., 1998). After N, P is the most frequently limiting macronutrient for plant growth (Schachtman et al., 1998). However, the high concentration of nutrients in shoots in the treatment without minerals and N addition (CNN) (table 5) indicates that there was no severe deficiency and therefore the nutrient demand from seedlings seemed small. Nevertheless, P is relatively immobile in soils (Walker and Syers, 1976), and an extensive root system should be advantageous for uptake of nutrients with low mobility (George, et al., 1997). Therefore, in the presence of a new source of P, plant growth increases and thus, the high increase in root length could be a response of the seedlings to improve P acquisition, since they will explore the soil more efficiently (Lynch, 1995). George et al. (1997) showed that fine roots of Scots pine respond to local sources with greater nutrient availability by increased root proliferation, and Treseder et al. (2007) also refer to increased root length as a means to acquire more P in the soil.

Ectomycorrhizal fungi produce a large mycelial surface area in contact with the soil and access nutrient sources unavailable to roots (Smith and Read, 2008). Although ECM fungi have long been associated with higher P uptake by plants in phosphorus poor soils, where external hyphae of mycorrhizal fungi can absorb P beyond the root-depletion zones (Colpaert et al., 1999), in the present study P may have inhibited ECM formation since in the apatite treatment very low mycorrhizal colonization was observed in comparison with the other treatments, based on a visual assessment at the 84 day harvest (figure 8). This suggests that P inhibited ECM root establishment. In contrast to the apatite, seedlings in the biotite treatment were mycorrhizal.

The effect of ectomycorrhizal symbiosis on plant growth will depend on the balance between the improvement of the host plant mineral nutrition and the additional carbon cost due to fungal metabolic activities (Plassard et al., 2000). This suggests that the additional carbon cost of the ECM fungi to the seedlings is necessary to access nutrient sources unavailable to roots however it did not improve plant growth significantly at day 84. This could be caused by the rapid growth of fungal hyphae in the substrate outside the roots, acting as a carbon drain (Plassard et al., 2000).

Table 4. P-values from a two-way analysis of variance (ANOVA) of elemental concentrations in soil solution and shoots, soil pH and plant growth in response to N and mineral amendments.

Parameters	N	Minerals	N x Minerals
Shoot dry weight (mg)	**0.003**	**0.000**	**0.000**
Root length (cm)	**0.000**	**0.000**	0.916
Elements in shoots (mg g^{-1})			
Ca	0.066	0.541	0.389
K	0.090	0.162	0.180
Mg	**0.000**	**0.000**	0.202
P	**0.000**	**0.000**	0.091
N	**0.000**	**0.000**	**0.012**
Elements in soil solution (mg l^{-1})			
Ca	**0.002**	**0.000**	0.108
K	**0.000**	**0.000**	0.092
Mg	**0.000**	**0.000**	**0.001**
P	0.362	**0.000**	0.408
Al	**0.000**	**0.000**	**0.021**
Fe	**0.000**	**0.000**	**0.000**
NH_4 - N	**0.000**	**0.031**	**0.008**
NO_3/NO_2 - N	**0.000**	**0.000**	**0.000**
Soil solution pH (H_2O) at days 0 and 84	**0.000**	**0.000**	**0.000**

Numbers in bold are significant.

Enhanced growth and yield is the most common reason to fertilize forests (Tiedemann et al., 1998). However, in the present experiment, N addition did not improve seedlings growth, at least during the initial growth period (84 days). One reason could be that N was not limiting growth. The effect of N addition on shoot biomass had a clear positive effect only in the apatite treatment, suggesting an interaction between P and N. In the other treatments there was a trend (although not significant) suggesting that increasing amounts of N resulted in lower shoot biomass (table 6). This was corroborated by the negative correlation between shoot N concentration and shoot biomass (figure 9; $r = -0.60$, $p < 0.001$).

Table 5. Concentrations (mg g^{-1}) of different elements in shoots 84 days after the beginning of the experiment.

Treatment	Elemental concentration (mg g^{-1})				
	Ca	K	Mg	P	N (%)
CNN	1,71 ± 0,34 a	5,90 ± 0,51 a	1,18 ± 0,04 ab	1,94 ± 0,09 bcd	3.67 ± 0.16 abc
CLN	1,24 ± 0,28 a	5,51 ± 0,64 a	0,75 ± 0,02 c	1,03 ± 0,15 fgh	3.34 ± 0.20 abcd
CHN	1,25 ± 0,23 a	5,03 ± 0,40 a	0,68 ± 0,04 c	0,81 ± 0,08 h	4.07 ± 0.10 a
QNN	1,14 ± 0,09 a	8,11 ± 0,69 a	0,95 ± 0,04 bc	1,75 ± 0,22 cdef	3.01 ± 0.11 bcd
QLN	1,49 ± 0,02 a	6,34 ± 0,55 a	0,90 ± 0,07 bc	1,63 ± 0,13 cdefg	4.04 ± 0.17 a
QHN	1,00 ± 0,15 a	4,07 ± 0,14 a	0,75 ± 0,09 c	0,94 ± 0,11 gh	3.73 ± 0.10 abc
ANN	1,65 ± 0,10 a	7,84 ± 0,14 a	1,37 ± 0,13 a	2,74 ± 0,31 a	2.56 ± 0.13 d
ALN	1,42 ± 0,10 a	6,64 ± 0,44 a	1,20 ± 0,02 ab	2,49 ± 0,05 ab	2.89 ± 0.09 cd
AHN	1,47 ± 0,06 a	8,07 ± 0,56 a	1,22 ± 0,12 ab	2,30 ± 0,06 abc	2.88 ± 0.24 cd
BNN	1,96 ± 0,63 a	7,95 ± 0,50 a	1,08 ± 0,11 abc	1,85 ± 0,13 bcde	2.95 ± 0.07 bcd
BLN	1,23 ± 0,22 a	6,47 ± 1,09 a	0,88 ± 0,11 bc	1,56 ± 0,12 defg	3.77 ± 0.33 ab
BHN	1,00 ± 0,16 a	6,81 ± 0,53 a	0,75 ± 0,04 c	1,18 ± 0,03 efgh	3.64 ± 0.14 abc

The seedlings were grown in forest soil with different levels of N addition (NN = No N; LN = Low N; HN = High N) and different minerals amendments (C = Control treatment with no minerals added; Q = Quartz; A = Apatite; B = Biotite). Values are means ± SEM of three replicates. Strong deficiency (mg g^{-1}): Ca <0.4, K < 3.5, Mg < 0.4, P < 1.2. Optimum level (mg g^{-1}): Ca > 0.7, K > 6, Mg > 0.8, P > 1.8 (Braekke and Salih, 2002). Significant differences among means were marked with different letters.

Table 6. Growth parameters of the pine seedlings under different N and mineral treatments, 84 days after the beginning of the experiment.

Treatment	Shoot dry weight (mg)	Root length (cm)
CNN	99 ± 6 d	152 ± 12 bc
CLN	112 ± 3 d	123 ± 20 bc
CHN	88 ± 7 d	80 ± 10 c
QNN	121 ± 12 d	175 ± 20 b
QLN	96 ± 8 d	112 ± 12 bc
QHN	72 ± 5 d	98 ± 2 bc
ANN	300 ± 10 c	327 ± 7 a
ALN	397 ± 20 b	276 ± 25 a
AHN	587 ± 51 a	271 ± 26 a
BNN	126 ± 11 d	139 ± 21 bc
BLN	98 ± 17 d	95 ± 18 bc
BHN	94 ± 4 d	69 ± 7 c

The seedlings were grown in pots containing forest soil with different levels of N addition (NN = No N; LN = Low N; HN = High N) and different minerals amendments (C = Control treatment with no minerals added; Q = Quartz; A = Apatite; B = Biotite). Values are means ± SEM of three replicates. Significant differences among means were marked with different letters.

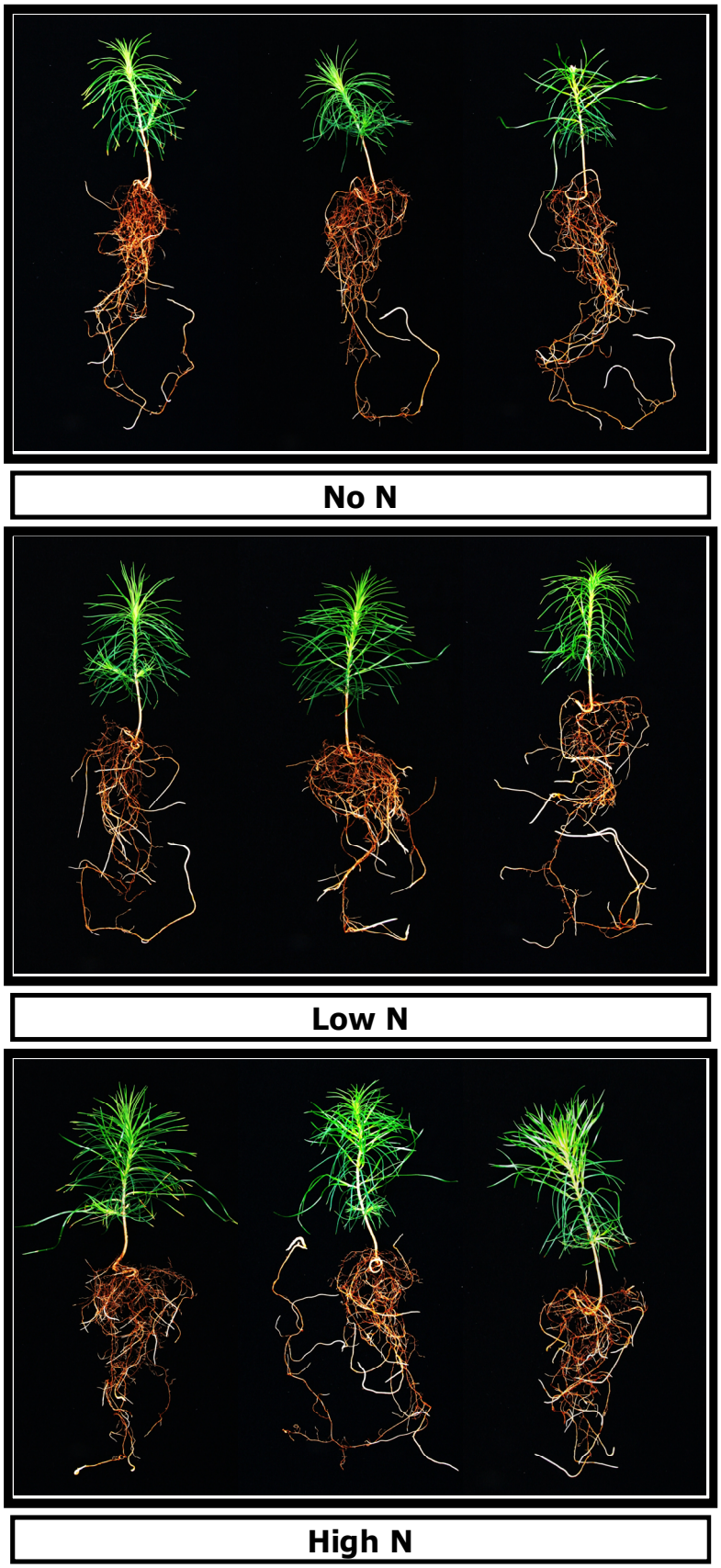


Figure 8. Pine seedlings harvested from triplicate apatite-amended microcosms with different levels of N addition. Seedlings demonstrate high growth and roots with reduced ECM colonization, 84 days after the beginning of the experiment.

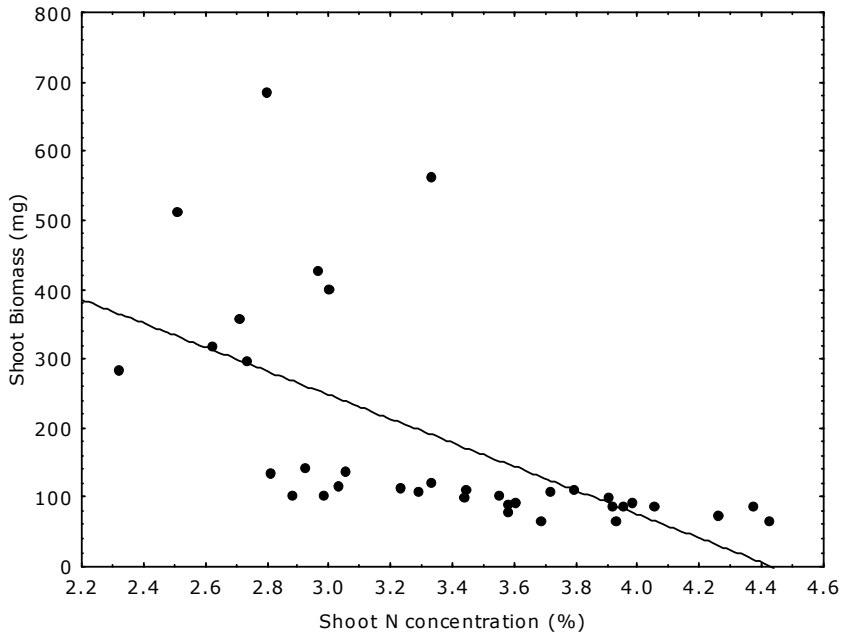


Figure 9. Relationship between shoot N concentration (%) and shoot biomass (mg) after 84 days of the beginning of the experiment ($r = -0.60$, $p < 0.001$).

No significant differences in root length were observed between N levels in the apatite treatment, although there was a tendency for lower values in the presence of N. In all other treatments there was a negative effect of N on root length (table 6). Tiedemann et al., (1998) mentioned that the application of a large amount of a single nutrient such as N has the potential to create limitations for other nutrients or even create deficiencies for nutrients in marginal supply. However, similar or even enhanced growth of coniferous species was observed in forests when N fertilization was supplemented with nutrients in deficiency, like P (Clarholm and Rosengren-Brinck, 1995). This is consistent with the demonstrated results, where N addition supplemented with apatite did not reduce plant growth compared to control, quartz and biotite, suggesting that N alone tended to limit P availability to seedlings but in the presence of a P mineral source the negative effect was not apparent.

If N fertilization limits uptake of other nutrients, then plants may invest more in ECM fungi and therefore allocate more carbohydrates to them to allow a greater absorption surface area and enhance nutrient uptake. Consequently, ECM fungi will benefit from nutrients with a higher tendency to become immobilized. More N retention by ECM fungi can result in reduced plant growth according to Nylund and Wallander (1989), Fransson et al. (2005), Alberton et al. (2007) and Alberton and Kuyper (2009). And in fact, all treatments that showed lower shoot biomass were mycorrhizal in contrast to the apatite treatment.

4.2.2 Weathering budgets

Different weathering budgets have been used to assess the mobilization of nutrients from minerals. The budget approach of Wallander and Wickman (1999), Wallander (2000a) and Wallander et al. (2005) was based on element concentrations in plants. Assuming the elements in plants derived from mineral weathering, Wallander and Wickman (1999) studied biotite dissolution based on K uptake and Wallander (2000a) and Wallander et al. (2005) studied apatite dissolution based on P uptake by plants. Van Hees et al. (2006) based their approach not only on plant uptake but also on the composition of drainage water and exchangeable cations in soil. Calvaruso et al. (2006, 2010) based their weathering budget on the composition of drainage solution and accumulation of elements in plants. Analyses of drainage solutions are also important because they allow a better assessment of mobilization of nutrients from minerals, even when they were not used by plants.

In the present experiment, mineral weathering was assessed by analysing nutrients in shoots and soil solution. However, this is an incomplete assessment because it does not take into consideration exchangeable cations that can easily become available to plants through ion exchange. Van Hees et al. (2006) mentioned that analyzing these cations is important in weathering assessment. Nevertheless, it was possible to draw some conclusions about the weathering of apatite and biotite, but a true quantification of the amount of mobilized elements was not possible.

The elemental composition of the soil solution provides a measurement of available essential elements because it represents the natural medium for plant growth (Arocena and Glowa, 2000). The process of nutrient uptake by plants refers to the transfer of the nutrient ions across the soil-root interfaces into the plant.

4.2.3 Mobilization of nutrients from apatite

Weathering in response to P or Ca does not seem to have occurred since according to Braekke and Salih (2002) the macronutrients analysed in shoots grown in the treatments with minerals but without N (CNN, QNN, ANN, BNN) were all above deficiency levels (table 5). This suggests that none of these elements were limiting growth. In the apatite treatment plant uptake of P and Mg was greater than in the other treatments (table 5).

Both P and Mg were significantly affected by N and minerals, but Ca was not significantly affected by N, minerals or the N x minerals interaction (table 4). The apatite treatment

stood out amongst other treatments because of the highest concentrations of elements mobilized to the soil solution, in particular Ca, Mg and P (table 7).

The P available to plants was only significantly affected by minerals, and Ca was only significantly affected by N and minerals. Soil Mg was significantly affected by N, minerals and the N x minerals interaction (table 4). The higher P uptake and concentration in soil solution in the apatite treatment, suggests that dissolution of apatite took place. Higher dissolution of primary P minerals, such as apatite, is usually associated with acidic conditions (Guindry and Mackenzie, 2003). Plant roots and microorganisms can alter solution P availability by acidification of the rhizosphere, exudation of organic acids, and secretion of extracellular phosphatases (Hinsinger, 2001; Vance et al., 2003). Rhizosphere acidification by release of protons and organic acids is thought to be the most important mechanism (Hinsinger, 2001; Hinsinger et al., 2003; Casarin et al., 2004). Therefore, changes in root architecture in response to apatite may be accompanied by rhizosphere acidification by protons and/or organic acids (Vance et al., 2003).

Soil pH was significantly affected by N, minerals and the N x minerals interaction (table 4) and the apatite treatment had the lowest pH, compared to control, quartz and biotite treatments (table 8). This suggests the importance of soil pH on the solubilization of P from apatite. However, a correlation between P in the soil and pH was not observed, probably because of the reduced effect of N in all treatments, but shoot P (as well as shoot Mg) was negatively correlated with soil pH (figure 10; $r = -0.74$, $p < 0.001$ and $r = -0.68$, $p < 0.001$, respectively). The increased uptake of these elements may also have contributed to the dissolution of apatite, mostly from the release of protons to counterbalance the cations entering the roots (Hinsinger, 2001; Hinsinger et al., 2003). The Ca and Mg present in soil solution were negatively correlated to soil pH (figure 11; $r = -0.51$, $p = 0.002$ and $r = -0.76$, $p < 0.001$, respectively).

Table 7. Concentrations (mg l^{-1}) of different macronutrients in soil solution collected from different N and mineral treatments 84 days after the beginning of the experiment.

Treatment	Elemental concentration (mg l^{-1})							
	Ca	K	Mg	P	Al	Fe	NH_4 - N	NO_3/NO_2 - N
CNN	15,89 ± 1,99 bcd	27,19 ± 3,81 abcd	4,40 ± 0,50 bc	9,39 ± 1,58 b	1,29 ± 0,22 f	0,41 ± 0,04 f	17.95 ± 2.63 c	19.25 ± 9.47 cd
CLN	11,82 ± 2,85 d	23,78 ± 4,45 bcd	2,71 ± 0,64 bcd	12,17 ± 2,65 b	3,37 ± 0,94 bcdef	2,17 ± 0,64 def	86.33 ± 2.91 bc	46.40 ± 1.31 bcd
CHN	13,10 ± 3,77 cd	20,61 ± 7,17 cd	2,38 ± 0,75 cd	11,74 ± 3,84 b	6,15 ± 1,31 ab	4,98 ± 1,04 b	106.53 ± 38.69 b	40.20 ± 12.97 bcd
QNN	23,12 ± 0,84 bc	39,40 ± 0,56 ab	6,70 ± 0,08 a	11,83 ± 0,50 b	1,58 ± 0,11 ef	0,62 ± 0,08 ef	18.33 ± 2.27 c	21.25 ± 9.46 cd
QLN	14,11 ± 2,17 cd	30,42 ± 5,42 abcd	3,21 ± 0,50 bcd	14,26 ± 1,88 b	4,13 ± 0,45 bcdef	2,83 ± 0,25 bcde	105.47 ± 27.44 b	50.80 ± 10.13 bcd
QHN	10,26 ± 1,16 d	14,94 ± 1,56 d	1,74 ± 0,17 d	9,39 ± 0,89 b	5,47 ± 0,46 bcd	4,66 ± 0,53 bc	77.00 ± 9.32 bc	27.27 ± 3.61 bcd
ANN	38,12 ± 1,58 a	33,97 ± 0,93 abc	8,12 ± 0,36 a	59,49 ± 1,76 a	2,45 ± 0,14 def	0,54 ± 0,02 ef	13.55 ± 3.cd62 c	15.75 ± 2.47 d
ALN	37,28 ± 3,31 a	32,77 ± 1,76 abc	7,83 ± 0,52 a	57,65 ± 2,92 a	2,68 ± 0,18 cdef	0,84 ± 0,03 ef	92.13 ± 6.12 bc	59.60 ± 6.31 b
AHN	37,30 ± 1,02 a	30,12 ± 1,81 abcd	7,13 ± 0,09 a	58,75 ± 2,84 a	4,60 ± 0,32 bcde	2,45 ± 0,28 cdef	197.00 ± 18.88 a	111.93 ± 9.96 a
BNN	26,17 ± 0,33 b	43,32 ± 0,14 a	7,42 ± 0,23 a	13,17 ± 0,65 b	2,04 ± 0,12 ef	0,82 ± 0,07 ef	21.49 ± 1.83 c	30.20 ± 2.50 bcd
BLN	20,45 ± 0,10 bcd	36,47 ± 0,98 abc	4,58 ± 0,09 b	18,22 ± 0,97 b	5,79 ± 0,21 bc	4,19 ± 0,15 bcd	113.50 ± 15.83 b	52.60 ± 0.76 bc
BHN	23,08 ± 0,19 bc	31,68 ± 0,59 abc	4,07 ± 0,11 bc	18,41 ± 0,47 b	9,27 ± 0,24 a	8,44 ± 0,84 a	155.53 ± 14.06 ab	54.67 ± 1.33 bc

The soil treatments consisted of different N levels (NN = No N; LN = Low N; HN = High N) and different minerals amendments (C = Control treatment with no minerals added; Q = Quartz; A = Apatite; B = Biotite). Values are means ± SEM of three replicates. Significant differences among means were marked with different letters.

Table 8. Soil solution pH in the different N and mineral treatments, at the start of the experiment and at day 84.

	Treatment	pH (H_2O)
0 days	CNN	3,87 ± 0,03 d
	CLN	3,92 ± 0,01 cd
	CHN	4,13 ± 0,02 aef
	QNN	3,94 ± 0,01 bcd
	QLN	4,07 ± 0,01 ef
	QHN	4,21 ± 0,03 a
	ANN	3,93 ± 0,02 bcd
	ALN	4,07 ± 0,02 ef
	AHN	4,14 ± 0,01 ae
	BNN	4,01 ± 0,04 bcf
	BLN	4,04 ± 0,03 bef
	BHN	4,10 ± 0,02 aef
84 days	CNN	3,86 ± 0,02 bd
	CLN	4,10 ± 0,01 c
	CHN	4,35 ± 0,04 a
	QNN	3,77 ± 0,03 d
	QLN	4,10 ± 0,02 c
	QHN	4,31 ± 0,02 a
	ANN	3,82 ± 0,04 d
	ALN	3,91 ± 0,03 bd
	AHN	4,01 ± 0,04 bc
	BNN	3,78 ± 0,04 d
	BLN	4,13 ± 0,02 c
	BHN	4,39 ± 0,03 a

The forest soil received different N levels (NN = No N; LN = Low N; HN = High N) and different minerals amendments (C = Control treatment with no minerals added; Q = Quartz; A = Apatite; B = Biotite). Values are means ± SEM of three replicates. Significant differences among means were marked with different letters.

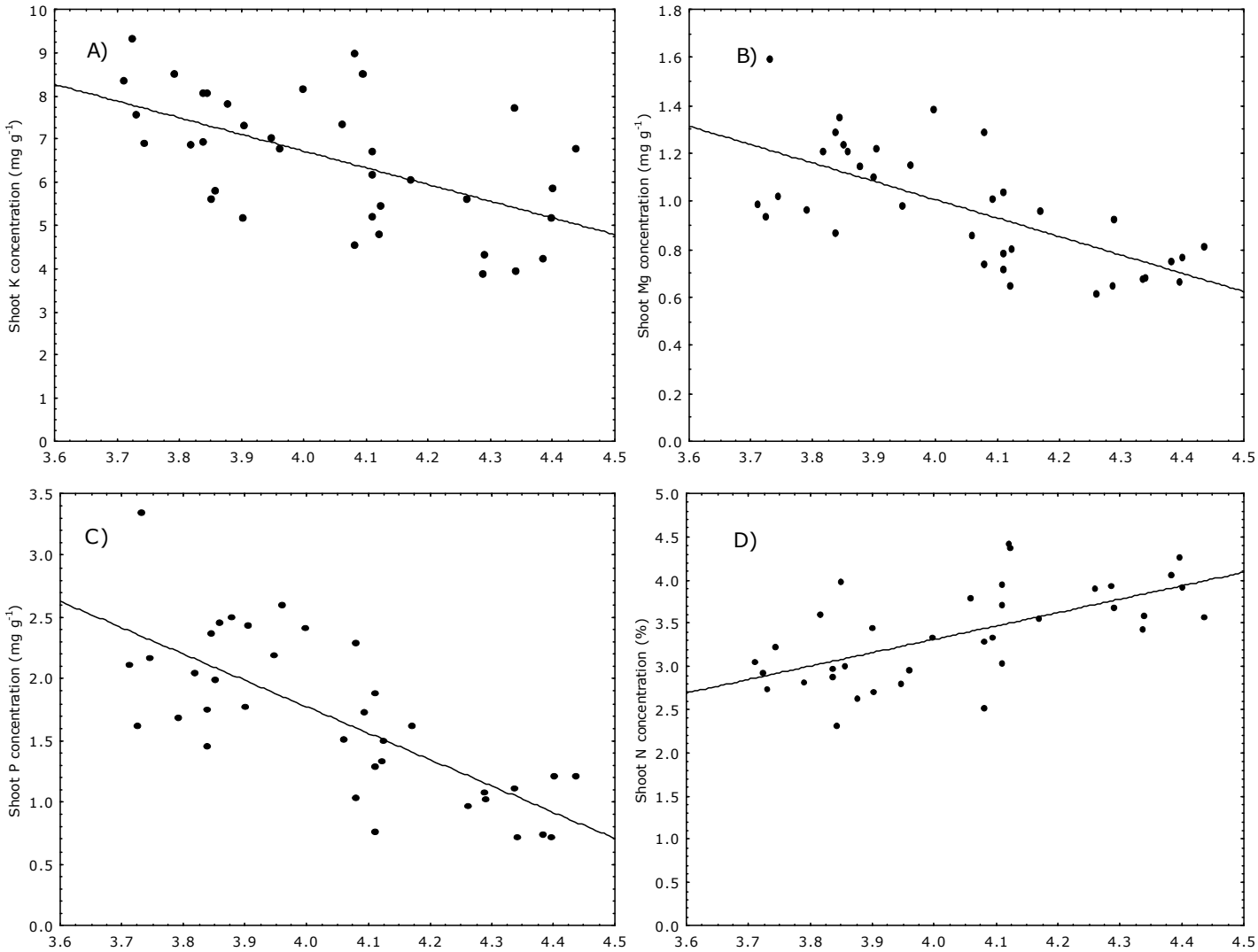


Figure 10. Relationships between concentration of A) K (r = - 0.57, p < 0.001), B) Mg (r = - 0.68, p < 0.001), C) P (r = - 0.74, p < 0.001) and D) N (r = 0.62, p < 0.001) present in shoots and soil pH by 84 days after the beginning of the experiment.

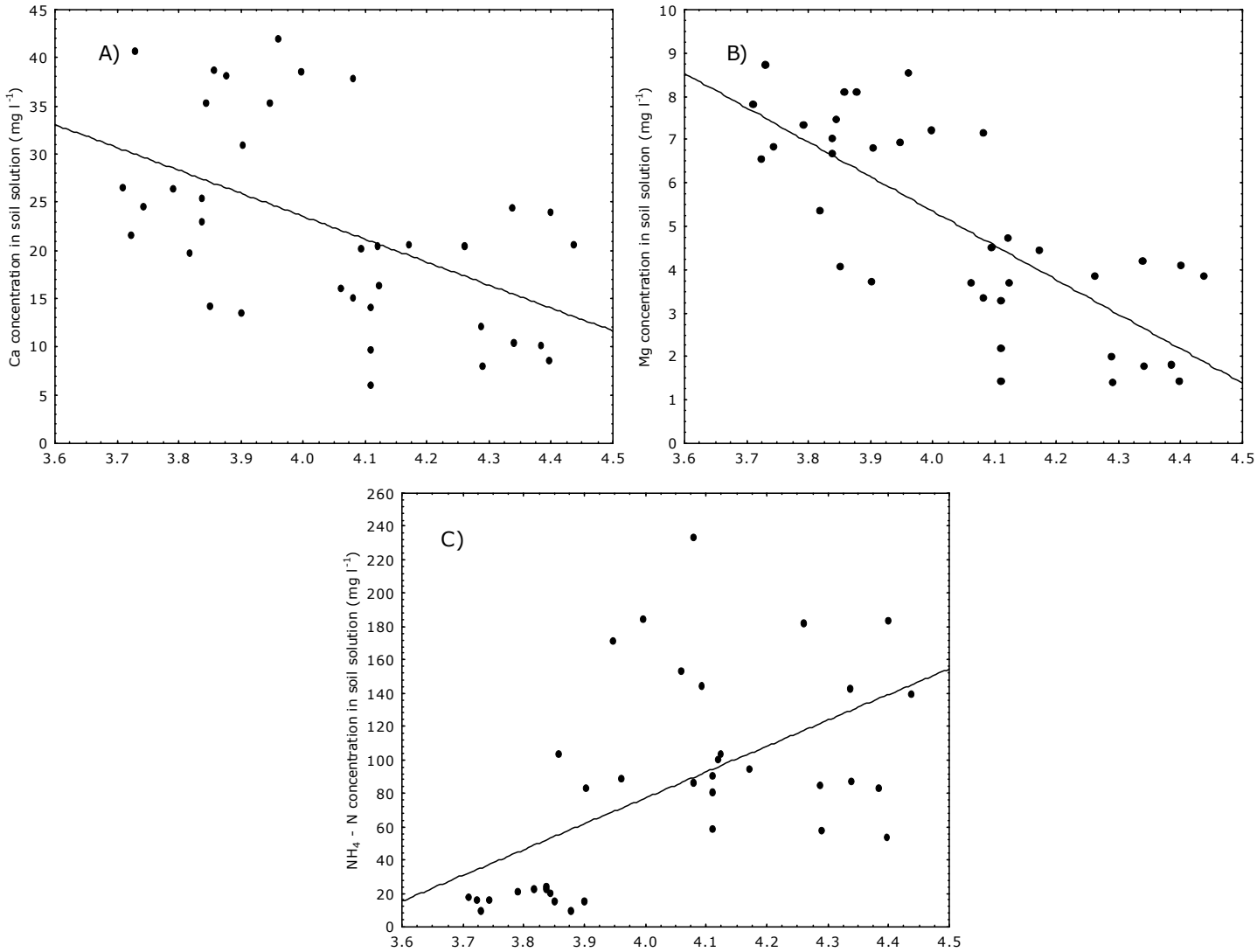


Figure 11. Relationship between concentration of A) Ca (r = - 0.51, p = 0.002) B) Mg (r = - 0.76, p < 0.001) and C) NH_4 - N (r = 0.56, p = 0.001) present in soil solution (mg l^{-1}) and soil pH by 84 days after the beginning of the experiment.

Seedlings in the apatite treatment were checked in the weeks following the 84 day harvest, and the pots were highly mycorrhizal (figure 12), perhaps due to the reduction in P with time. Fungi influence mineral dissolution through nutrient uptake by the mycelia and through acidification and chemical processes induced by their exudates (Rosling, 2009). Since P is relatively immobile in soils (Hinsinger, 2001), the uptake of P generates a depletion zone (Vance et al., 2003) and therefore P acquisition is favored by mycorrhizal uptake as hyphae bridge the depletion zone (Vance et al., 2003). Several studies have shown that apatite addition has a positive influence on ECM development in forests with limiting P but not with sufficient P (Hagerberg et al., 2003; Nilsson and Wallander, 2003; Wallander and Thelin, 2008).

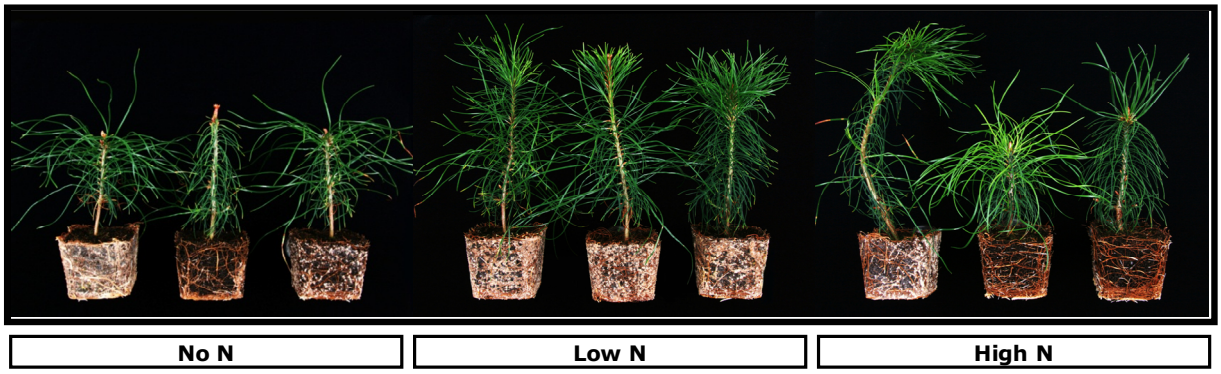


Figure 12. Pine seedlings (*Pinus sylvestris* L.) in the apatite treatment with different levels of N addition, a few weeks after the 84 day harvest. The seedlings showed a developed mycorrhizal colonization.

4.2.4 Mobilization of nutrients from biotite

Biotite, a common K- and Mg-containing mineral in soils, was not strongly weathered. According to Brakke and Salih (2002), K and Mg were not limiting plant growth in the control and in biotite treatments (CNN and BNN) as their concentrations were within the optimum range. No significant increase in nutrient uptake was registered (table 5). The K concentration in shoots was not significantly affected by N, minerals or the N x minerals interaction (table 4). However, the K concentration was higher in the biotite treatment than in the control. This suggests that some mineral dissolution might have occurred.

Plant growth did not respond to biotite but a non-significant trend was observed for a reduced root length compared to the control, as well as for a slightly higher shoot K concentration.

Only K, Al and Fe were in higher concentrations in soil solution in the biotite treatment compared to the other elements (table 7) although not in the same proportion as P in the apatite treatment. In soil solution, K was significantly affected by N and minerals and Al and Fe by N, minerals and the N x minerals interaction (table 4). Even though weathering of biotite was limited, additional amounts of Al and Fe in the soil solution also suggest some mineral mobilization.

Mycorrhizal colonization was observed in the biotite treatment (figure 13), as well as in control and the quartz treatments. However, no assessment of ectomycorrhizal colonization was made and visual observation alone did not enable conclusions to be drawn about differences in the degree of mycorrhization between different treatments. Unexpectedly, Mg mobilization from biotite was not stimulated, as there was no increase in Mg uptake, with only a small increase in Mg in soil solution compared to control, but less than in the apatite treatment. According to Wallander and Wickman (1999) and Jentschke et al. (2001) the presence of mycorrhizal colonization does not increase Mg uptake.

The potential of ectomycorrhizal fungi to dissolve and take up K has been studied mainly using mica minerals (biotite, muscovite and phlogopite) (Rosling, 2009), but the role of mycorrhiza in K uptake is uncertain, with contrasting results in both laboratory and field experiments. Ekblad et al. (1995) and Wallander and Wickman (1999) found that K deficiency decreased the ECM fungal biomass from *Pinus sylvestris* which might reflect reduced carbon allocation to roots. The ECM fungi have been shown to improve the uptake of K derived from biotite weathering (Wallander and Wickman, 1999; Wallander, 2000b; Calvaruso et al., 2010). However, Hagerberg et al. (2003) in a mesh bag experiment in the field showed that in a boreal forest ECM fungi had a small potential to improve the amount of K by mineral weathering because no stimulation of root colonization or ECM mycelia production was observed even when shoot K was small. Furthermore, different ECM fungal species appeared to have a differential influence on weathering of biotite in a laboratory pot system (Wallander, 2000b). Van Schöll et al. (2006) also demonstrated that ectomycorrhizal fungi may increase weathering of minerals in response to nutrient deficiencies, but this response is species specific.

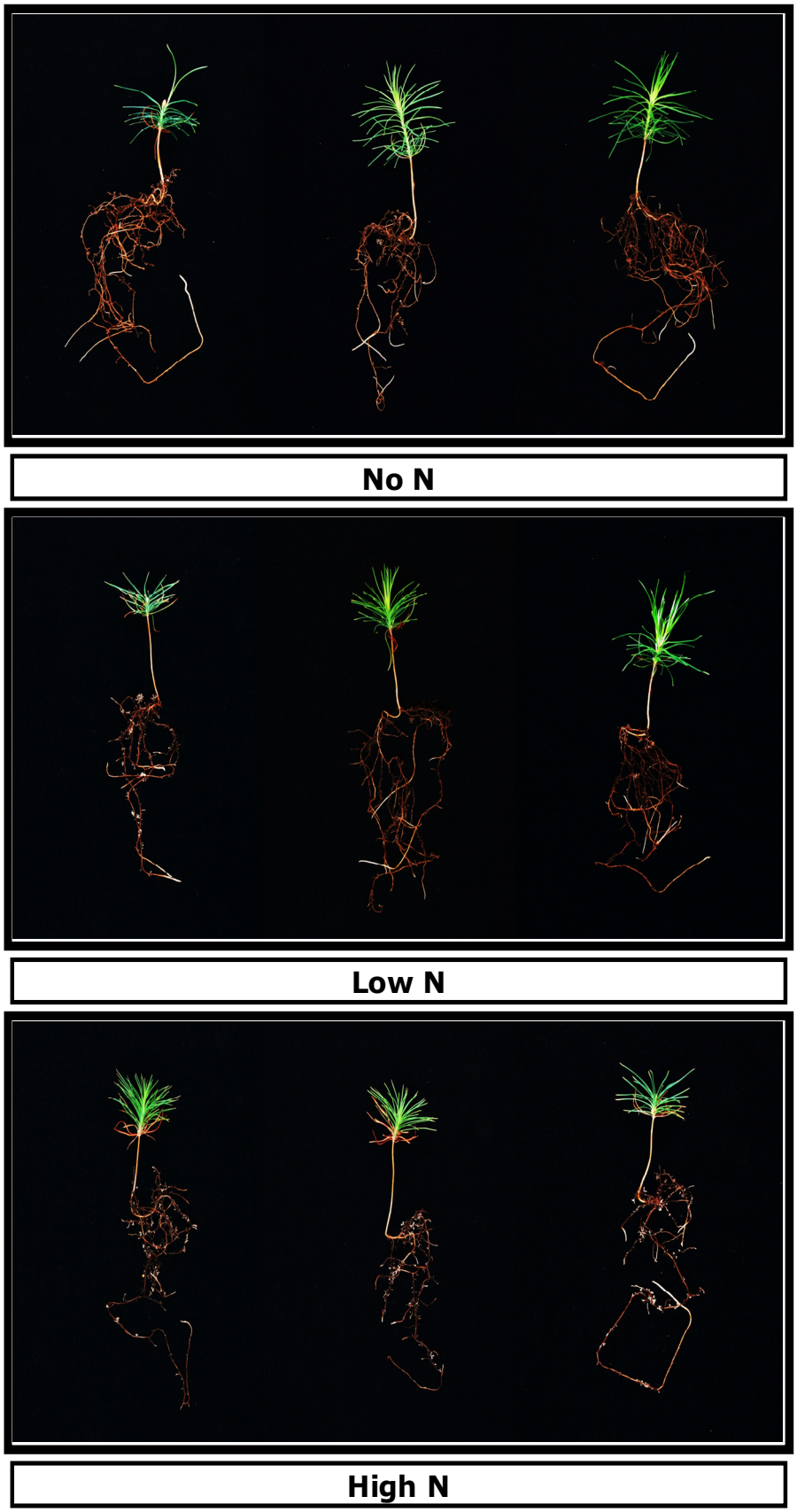


Figure 13. Pine seedlings harvested from triplicate biotite-amended microcosms with different levels of N addiction. Seedlings demonstrate roots with ECM colonization, 84 days after the beginning of the experiment.

4.2.5 Conclusions

Apatite had a stimulatory effect on the growth of seedlings although P in the soil was apparently not a limiting factor. No indication was found that P derived from weathering was dependent on ECM symbionts, at least until day 84. Rosling (2009) pointed out that P uptake can be the main biotic mechanism of weathering of primary P minerals, since the dissolution of the minerals is sufficient to buffer P concentration in the soil solution (Oelkers et al., 2008). No significant stimulatory effect of the biotite treatment was found on seedlings growth, but a relative low mobilization of elements from biotite seemed to take place, although much less than in the apatite treatment. This might be explained by the slower dissolution rate of biotite compared with apatite (Hagerberg et al., 2003).

In this experiment, 53.4 mg of K and 42.4 mg of Mg were added to the soil in the biotite while in apatite 238.5 mg of Ca and 143.6 mg of P were present. The referred amounts of macronutrients were added to the soil because of the different proportions already existing in biotite and apatite, and this should be considered when assessing dissolution rates. Usually biotite has been used in higher amounts (e.g. 3% (w/w)) compared to apatite (1% (w/w)) in several pot systems and mesh bag studies (Wallander et al., 1997; Wallander and Wickman, 1999; Wallander, 2000a; Wallander et al., 2005; Wallander et al., 2006). Nevertheless, we should consider that the dissolution rate of minerals may be higher in the present experiment compared to the natural environment due to the small volume available for root development in the pots.

Quartz is known to be highly resistant to weathering and an inert mineral. Thus, it was expected to produce results similar to those of the control treatment. However, slightly higher concentrations of elements analysed were present in soil solution and in shoots compared to the control treatment, suggesting that some weathering did take place.

4.3 EFFECT OF N ON MOBILIZATION OF NUTRIENTS FROM PRIMARY MINERALS

Apatite and biotite have been studied as alternatives to fast release fertilizers to improve nutrient availability in soils (Aarnio and Martikainen, 1994; Aarnio et al., 1995). They are known to slowly increase nutrient availability to plants without major losses due to leaching (Aarnio et al., 1995). Nutrients from apatite and biotite are released gradually and enhanced availability of P, K, Mg and Ca is maintained in soils for long periods (Aarnio et al., 2003). Aarnio et al. (2003) reported a favorable effect could still be detected ten years after the combined application of apatite and biotite on the soil.

In the present study, N was added to the soil in the form of methylene urea, a common slow release N fertilizer. Methylene urea is a condensation product of urea and formaldehyde consisting of polymers with various chain lengths (Koivunen and Horwath, 2005). Degradation and subsequent release of plant available forms of N is controlled by its solubility, which depends on the degree of polymerization, and the combination of different polymers in the product (Koivunen and Horwath, 2004). The doses of application of methylene urea were chosen to follow the practice used in forest fertilization.

The complex urea-containing compounds are degraded by microorganisms before N becomes available. Hence, the hydrolysis of urea and nitrification of ammonium can alter soil pH. Nitrogen in shoots was significantly affected by N, minerals and the N x minerals interaction as well as NH_4-N and NO_3/NO_2-N concentrations in soil solution (table 4). Methylene urea added to the soil was converted to NH_4^+, leading to an increase in soil pH and NH_4-N concentration. Soil pH and NH_4-N concentrations were positively correlated (figure 11; $r = 0.56$, $p < 0.001$). Indeed, conifers are viewed as strongly adapted to utilize NH_4^+ probably because they are typically associated to acidic soils, with low rates of nitrification (Rothstein and Cregg, 2005). However, NN treatments (CNN, QNN, ANN, BNN) resulted in a reduction in the soil pH (table 8). In fact, in these treatments nitrification products (NO_3^-/NO_2^-) were somewhat higher than ammonium (table 7) compared to the N treatments. The relatively low amount of NO_3/NO_2-N measured in these treatments implies that the NO_3^-/NO_2^- produced was quickly immobilized. Also, after a subsequent increase in the NH_4-N concentration in all other N treatments, nitrification proceeded (especially in the apatite treatment, where the highest amounts of NO_3/NO_2-N were present), resulting in a decrease in pH. This indicates that methylene urea in the presence of apatite stimulated nitrification more than in other mineral treatments.

An increase in N uptake with N addition was observed. However, a lower shoot N concentration was detected in the apatite compared to control, quartz and biotite

treatments (table 5). Furthermore, NH_4-N and NO_3/NO_2-N concentrations in soil solution were not correlated with N uptake by the seedlings. However, shoot N was positively correlated with soil pH (figure 10; $r = 0.62$, $p < 0.001$) which suggests that some acidification process other than nitrification was taking place thereby affecting N uptake.

4.3.1 Effect of nitrogen on the mobilization of nutrients from apatite

Methylene urea did not have a significant effect on the weathering of apatite since there were no significant differences in P and Ca in this treatment (table 7). When the effect of N was tested separately on the apatite treatment to see if there was a change in the mobilization/uptake of nutrients, again no significant differences were found, except for the concentrations of Al and Fe in the soil (table 9). Aarnio et al. (1995) applied urea (46% N) together with apatite to a forest soil, and showed that soluble P in the soil decreased after three growing seasons when urea was applied. They stated that the increase in soil pH caused by the urea might slow down the weathering of apatite and increase microbial activity which would lead to the immobilization of P. Aarnio et al. (2003) in a similar experiment, but after 10 years of fertilizer application, demonstrated that urea (46% N) added together with apatite did not change soluble P compared to apatite alone. Soil pH was considered the main factor for apatite solubilisation since the lowest pH was observed in the apatite and apatite treated urea.

We should keep in mind that urea is a fast-release fertilizer compared to methylene urea and therefore a more abrupt change in soil pH would occur in response to urea addition that might reduce apatite weathering. However, after 10 years the effect of N was no longer apparent (Aarnio et al., 2003). In the present experiment, a slow release fertilizer was applied (38% N) and the data available at day 84 resemble those in the field experiment described by Aarnio et al. (2003). The effect of methylene urea on the soil pH in the apatite treatment decreased with time and no significant difference in available P was detected between N treatments and the NN treatment. Is possible to conclude that soil pH was important for P mobilization from apatite and in fact, P was the only soil element positively correlated to NO_3/NO_2-N concentrations in the soil (figure 14; $r = 0.46$, $p = 0.005$), which may have contributed to soil acidification. However, we should keep in mind that changes in soil pH also change the microbial community and its activity in the soil, and these in turn may affect the release of nutrients from minerals.

Table 9. P-values from a one-way analysis of variance (ANOVA) for elemental concentrations in soil solution and shoots in response to N levels in the apatite treatment.

Parameters	P
Elements in shoots (mg g^{-1})	
Ca	0.233
K	0.104
Mg	0.499
P	0.304
N	0.338
Elements in soil solution (mg l^{-1})	
Ca	0.954
K	0.275
Mg	0.227
P	0.880
Al	**0.001**
Fe	**0.000**

Numbers in bold are significant.

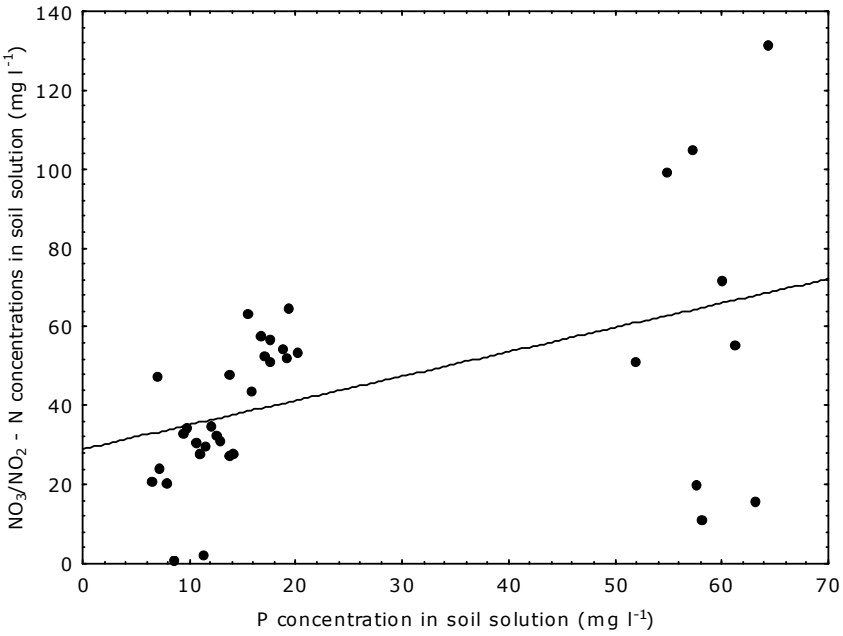


Figure 14. Relationship between NO_3/NO_2 – N and P concentration in soil solution (mg l^{-1}) at day 84 of the experiment (r = 0.46, p = 0.005).

4.3.2 Effect of nitrogen on the mobilization of nutrients from biotite

Methylene urea decreased K and Mg mobilization significantly in the biotite treatment but increased Al and Fe mobilization (table 7). When the effect of N was tested with data

from the biotite treatment to see whether there was a clear change in the mobilization/uptake of nutrients, only shoot P was significantly affected, but soil elements were all significantly affected by N addition (table 10).

Table 10. P-values from a one-way analysis of variance (ANOVA) of elemental concentrations in soil solution and shoots in response to N levels in the biotite treatment.

Parameters	P
Elements in shoots (mg g^{-1})	
Ca	0.271
K	0.411
Mg	0.110
P	**0.012**
N	0.070
Elements in soil solution (mg l^{-1})	
Ca	**0.004**
K	**0.000**
Mg	**0.000**
P	**0.004**
Al	**0.001**
Fe	**0.000**

Numbers in bold are significant.

Nitrogen addition decreased significantly shoot P concentration and a similar trend (although not significant) was observed for the other nutrients. Nitrogen also led to decreased Ca, K and Mg concentrations in the soil solution, while it increased P, Al and Fe concentrations. The high concentration of Al (> 4 mg l^{-1}; Arocena and Glowa, 2000) in the soil solution may have contributed to a reduction in P uptake. Phosphate ions can readily precipitate with Fe and Al into secondary minerals under acidic conditions (Hinsinger, 2001) and thus become unavailable to plants. The Fe and Al phosphates have an increasing solubility with increasing pH (Hinsinger, 2001; Hinsinger et al., 2003) and since methylene urea increased soil pH, it also increased Al, Fe and P concentrations in the soil solution. Phosphorus is also immobilized by sorption on Al and Fe oxides, especially at low pH (Hinsinger, 2001). This process could contribute to a decreased mobility of P in soil solution in the NN treatments, where the pH was lower. However, the release of exudates containing organic acids into the rhizosphere can lead to metal chelation and subsequent displacement of P from bound or precipitated forms, with a concomitant increase in its availability (Vance et al., 2003).

The negative effect of N on mobilization would occur in all treatments, but in the apatite treatment the reduction was small and therefore not significant. The positive effect of N on Al and Fe mobilization was significant even in the apatite treatment. The same did not happen with P concentration that did not increase in the apatite treatment but only in the others. Concentrations of available Al and Fe in soil solution were lower in the apatite treatment, probably due to a higher precipitation of Fe and Al phosphates due to the lower pH. Ion competition with NH_4^+ for uptake by the plant might also occur, particularly in the biotite treatment.

4.3.3 Effect of N on the uptake of nutrients

Shoot P in treatments without a P source (control, quartz and biotite) corresponded to a strong deficiency of the element (CLN, CHN, QHN, BHN). It seems that soil P had a small availability, and in the small pots used nutrient starvation could be easily reached compared to field conditions. Nitrogen application enhanced P deficiency, particularly in the control treatment, where no extra source of nutrients was present. This was consistent with the mycorrhizal status of the mineral treatments referred earlier. The higher P availability in the soil with apatite may have temporarily inhibited ECM formation compared to the biotite treatment, where the soil P concentration was lower. In biotite treatment, N addition intensified P deficiency and mycorrhizal formation was observed especially in the LN and HN treatments (figure 15). Several studies showed that N can cause P deficiency in forest stands (Rosengren-Brinck and Nihlgård, 1995; Teng and Timmer, 1995; Gundersen, 1998) as a result of fertilization or due to deposition of atmospheric N (Mohren et al., 1986; Houdijk and Roelofs, 1993; Stevens et al., 1993; Fenn et al., 2006; Akselsson et al., 2008). Wardle et al. (2004) showed that the transition from N to P limitation in ageing ecosystems is a natural process, and fungi become increasingly more important than bacteria as nutrient pools decrease. Perring et al. (2008) used a plant-soil nutrient model to study the mechanisms of P responses to N addition. It showed different responses according to specific pathways by which nutrients are lost from the ecosystem. Retention of P is promoted if the loss of plant available P is greater than the loss of less readily available organic P. However, P declines in response to N addition when the loss of less readily available organic P is greater than the loss of plant available P. In the present experiment, the soil used had large organic P and N contents (Giesler et al., 2002). Consequently, the P available to plants in the control, quartz and biotite treatments was mainly organic P. It has been proposed that the activity of phosphatase, which decomposes organic P, may increase at low concentrations of P, which would decrease the risk of P shortage for trees (Vance, 2001). When N is

added to the soil, loss of organic P is greater and the system becomes deficient. In the apatite treatment the P originating from the mineral contributed to a greater P uptake.

Shoot Mg concentration was within the optimum level in all treatments without N and in the apatite treatment. Even with N application, shoot Mg concentrations were close to the optimum (CLN, CHN, QHN and BHN), maybe because the concentration of Mg in soil solution was high. Shoot K was also within the optimum levels in the apatite and biotite, as well as in the QNN and QLN treatments. In the control treatments (CNN, CLN, CHN) the concentration was close to optimum, and it was only in the QHN treatment that the level of K was in the deficiency range.

Shoot Ca concentration was within optimum levels in all treatments. This indicates that the effect of N on nutrient uptake was less negative in the systems with increased availability of elements in mineral forms (particularly phosphate), compared to organic forms already present in the soil (control treatment with no minerals added).

Shoot K, Mg and P concentrations were negatively correlated with soil pH (figure 9) and shoot N (figure 16). This was due to a negative effect of N on the uptake of K, Mg and P. This effect was less evident at reduced pH, as the solubilisation of nutrients was higher in this case. A good example is the apatite treatment, with the lowest pH and highest uptake of nutrient except N. However, none of the elements analysed in shoots were correlated with NH_4-N or NO_3/NO_2-N concentrations in soil solution, although there was a trend for decreased uptake of all nutrients with increasing NH_4-N concentration in the soil, suggesting that smaller root length at high NH_4-N levels may influence nutrient uptake (Rothstein and Cregg, 2005).

Total plant contents of Ca, K, Mg and P increased with apatite addition (figure 17). This was expected since the apatite treatment was associated with the greatest seedling growth. Nitrogen application had a positive effect on shoot growth in this treatment as opposed to the control, quartz and biotite treatments.

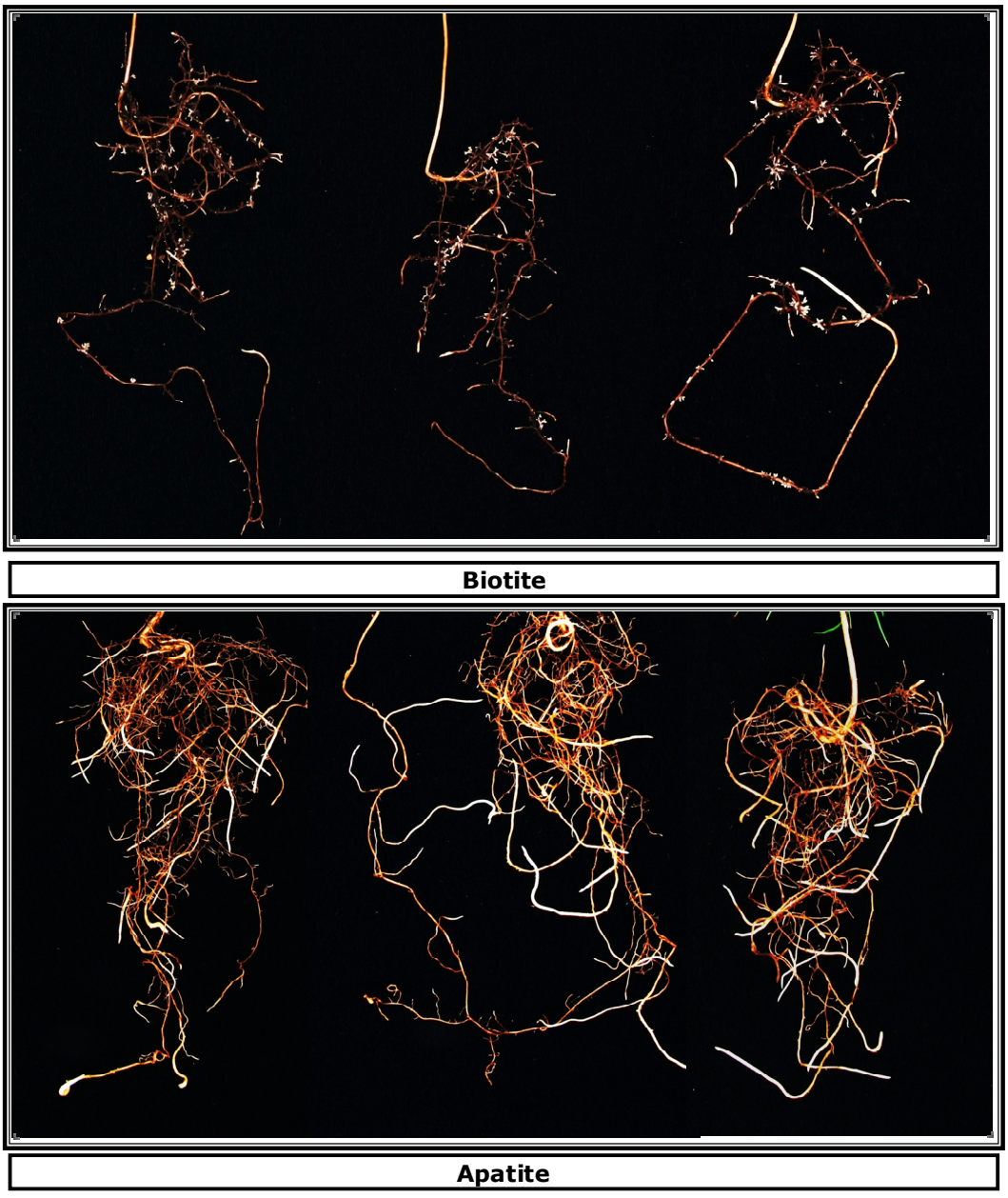


Figure 15. Varying degrees of ECM colonization in roots of pine seedlings from the biotite and apatite treatments with a high level of N addition at day 84.

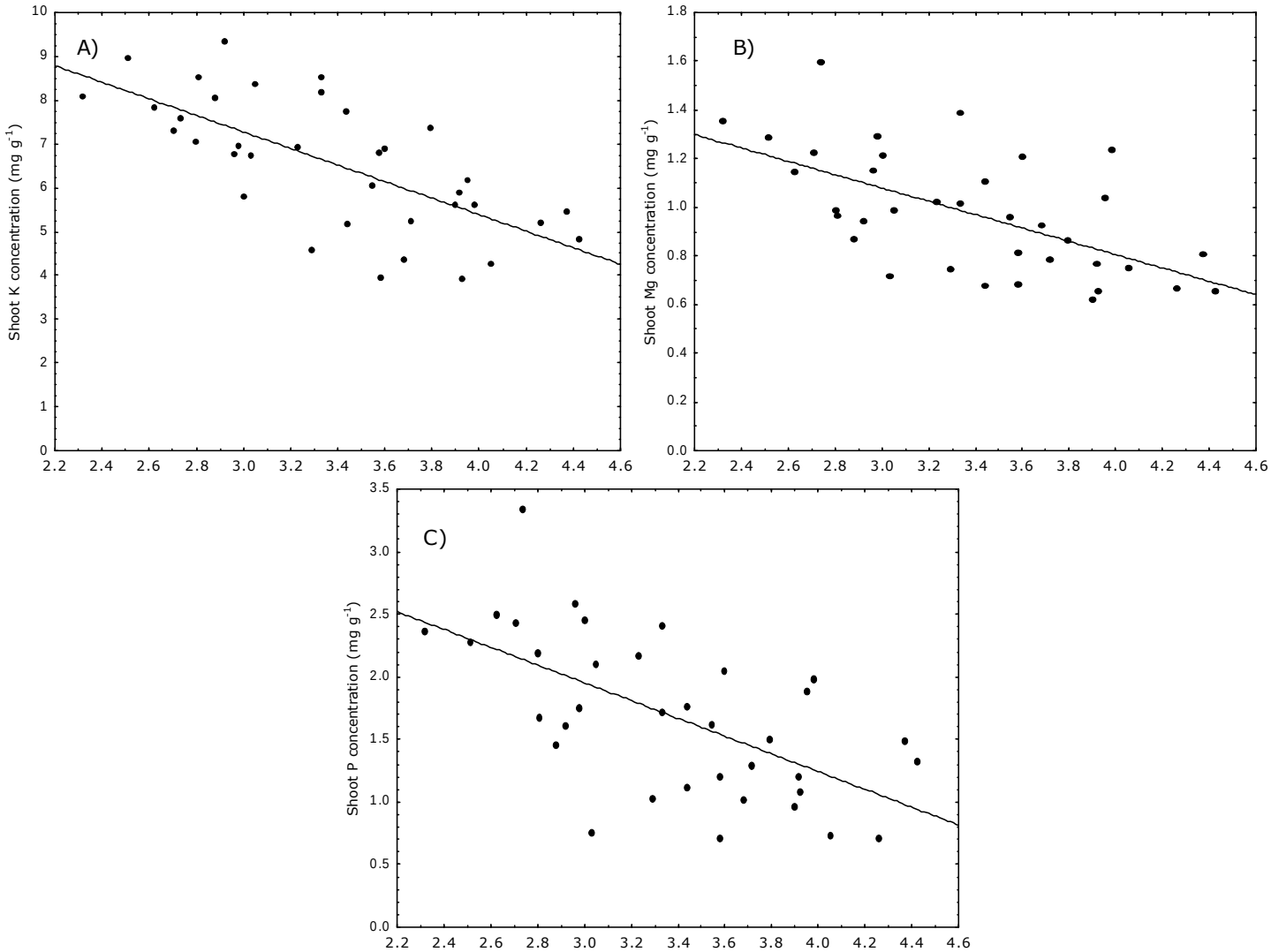


Figure 16. Relationship between shoot concentration (mg g^{-1}) of A) K ($r = -0.70$, $p < 0.001$), B) Mg ($r = -0.61$, $p < 0.001$) and C) P ($r = -0.61$, $p < 0.001$) and shoot N concentration (%) by 84 days after the beginning of the experiment.

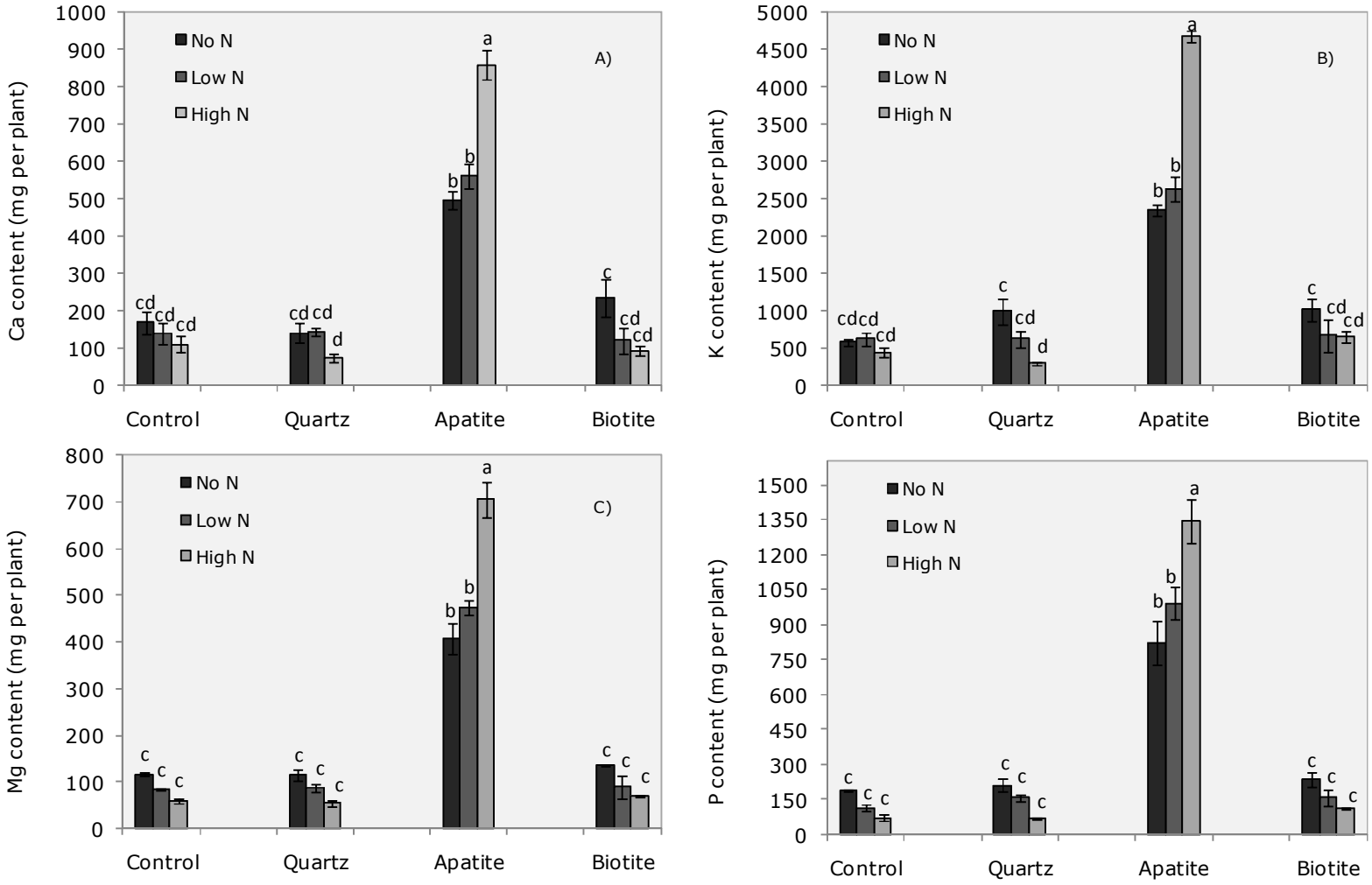


Figure 17. Content (mg per plant) of macronutrients in pine shoots grown with primary minerals (quartz, apatite, biotite or no mineral) and two concentrations of a slow release N fertiliser. A) Ca, B) K, C) Mg and D) P. Vertical bars represent SEM of three replicates. Significant differences among means are marked with different letters.

4.3.4 Conclusions

This experiment shows that the study of the effect of N fertilization on the weathering of primary minerals is of great importance in forest ecosystems. Nitrogen induced P depletion although the total amount of soil P was not in the deficiency range (Schachtman et al., 1998; Giesler et al., 2002). More data on microbial communities, in particular the fungal community, is required to improve the knowledge on the role played in nutrient mobilization since, (ECM) fungal nutrient demands and deficiencies may also lead to increased mineral weathering. Soil pH plays a dominant role in determining the composition of the microbial community (Högberg et al., 2007) but it is also affected by on-going processes like cation–anion balances, root exudation and respiration and redox-coupled processes (Hinsinger et al., 2003).

It was stated that the effects of N on ECM fungal communities may be stronger in nutrient-poor sites than in richer sites (Kjøller and Clemmensen, 2009). The results presented in this experiment are from relatively short-term interactions and therefore the conclusions made so far do not take into account possible biological and chemical changes over a longer period of time. In this microcosm experiment, the status of the plant-soil system changed towards a reduction of nutrients availability, and therefore less differentiation might be present in fungal community structure between mineral and N treatments on a longer time scale.

5 GENERAL CONCLUSIONS

This thesis demonstrates that:

- Nitrogen may increase ectomycorrhizal fungi abundance in the soil, as well as apatite.

- The root colonization by ectomycorrhizal fungi is reduced by increased availability of mineral P in the soil.

- Biotite has a much slower dissolution rate compared to apatite.

- Nitrogen induces reduction of nutrients uptake and mobilization into the soil solution.

Together these findings support the idea that nitrogen may have important effects on the fungal community involved in the mobilization of nutrients from primary minerals in the soil.

6 FUTURE PROSPECTS

The differences in basidiomycete communities structure observed in association with N fertilization emphasize the need to study the functions of different soil microorganisms. In particular, the importance of N-responsive taxa for weathering processes must be determined in order to attribute changes in mobilization of nutrients to shifts in the ECM community structure. These shifts in ECM communities may reflect differences in the functional abilities of ECM species to acquire nutrients from the different minerals present in soils. However, the interpretation of the functional significance of changes in community structure is still constrained by a lack of knowledge of the functional capabilities of most ECM taxa (Nygren, 2008).

Despite their importance and diversity, the taxonomic identity of the microorganisms involved in any specific process has usually been confined to the small fraction of the microbiota that has been isolated and cultivated (Radajewski et al., 2000). Therefore, one of the biggest challenges is to identify which microorganisms are carrying out a specific set of metabolic processes in a natural environment. Stable isotope probing (SIP) is a technique that is used to identify the microorganisms in environmental samples that use a particular growth substrate (Dumont and Murrel, 2005). It is based on labelling certain types of microbial biomarkers (DNA and RNA are the most informative taxonomic biomarkers) with stable isotopes (typically ^{13}C), followed by an analysis of the labelled biomarker pools to identify the members of the microbial community active in assimilating the substance of interest (Sims, 2007) by the selective recovery and analysis of isotope-enriched cellular components (^{13}C-labelled molecules) via density-gradient centrifugation (Dumont and Murrel, 2005). DNA-based SIP (DNA-SIP) is increasingly being used to link the identity of microorganisms to their functions. The most important feature of DNA-SIP is that the heaviest DNA (^{13}C-DNA) fraction collected following density-gradient centrifugation contains the combined genomes of a microbial population that is able to incorporate the labelled substrate into their nucleic acids (Radajewski et al., 2003).

SIP provides valuable information in rhizosphere–microorganism interactions (Griffiths et al., 2004; Singh et al., 2004; Rangel-Castro et al., 2005). One future approach to continue this experiment is to incubate plants with $^{13}CO_2$ and subsequently extract nucleic acids from the rhizosphere soil. Isolation of ^{13}C-DNA from this soil yields DNA to use as a template in PCR and to analyze ITS rDNA genes by DGGE analysis. This will give important information about rhizosphere fungal populations that are sequestering carbon from colonized roots in different mineral treatments with different N levels and also to discern about possible complex differences between treatment-specific communities.

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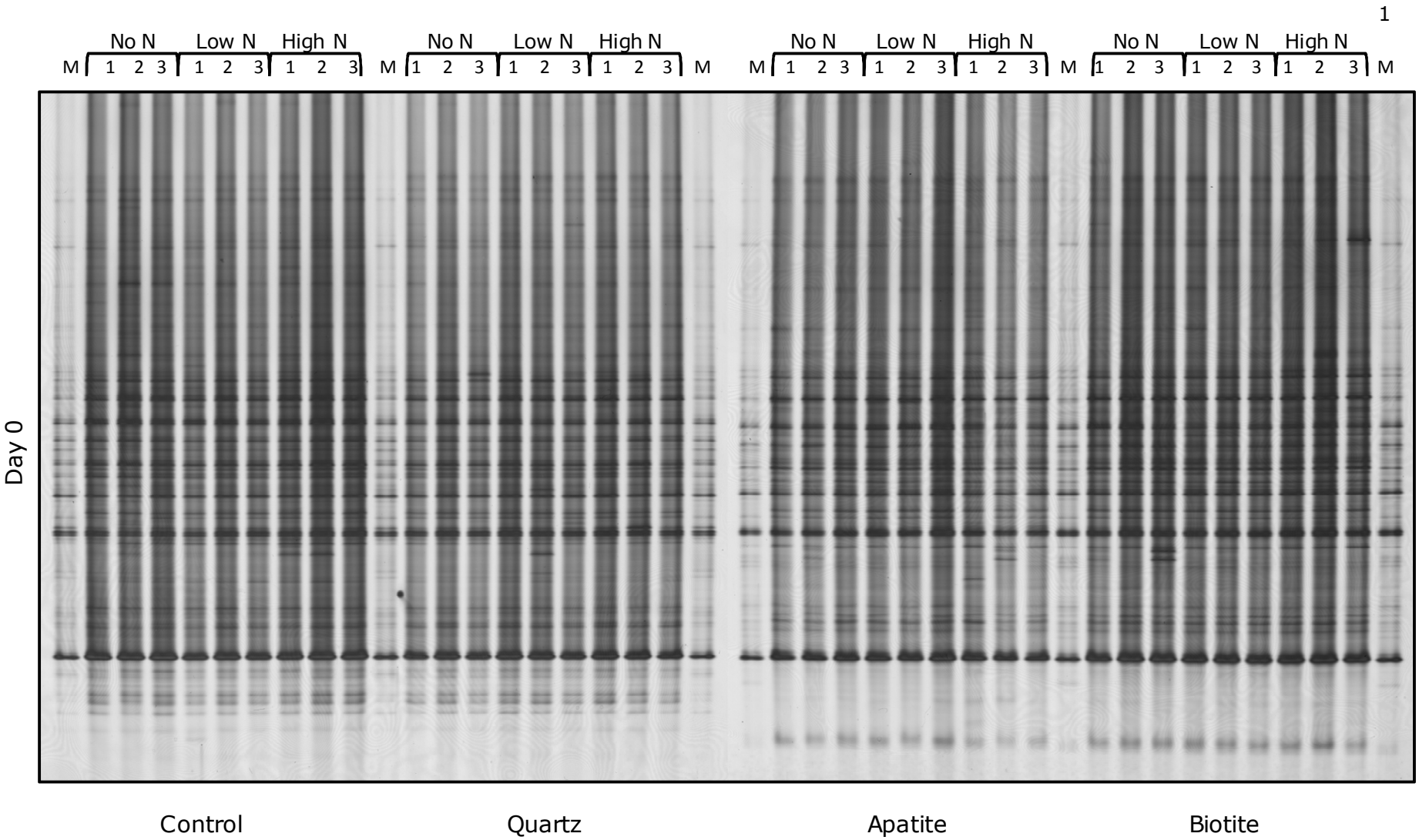
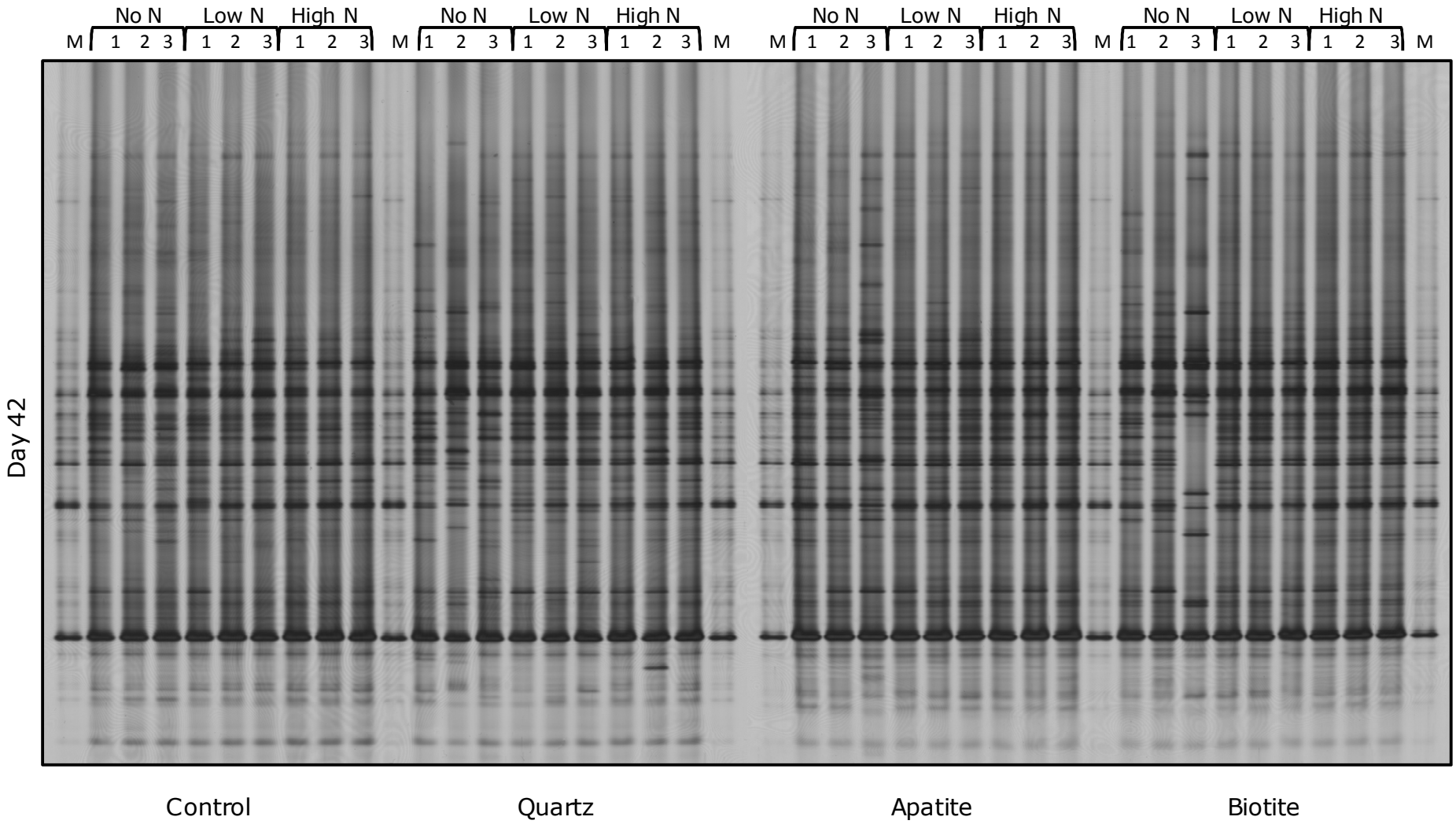
APPENDIX A

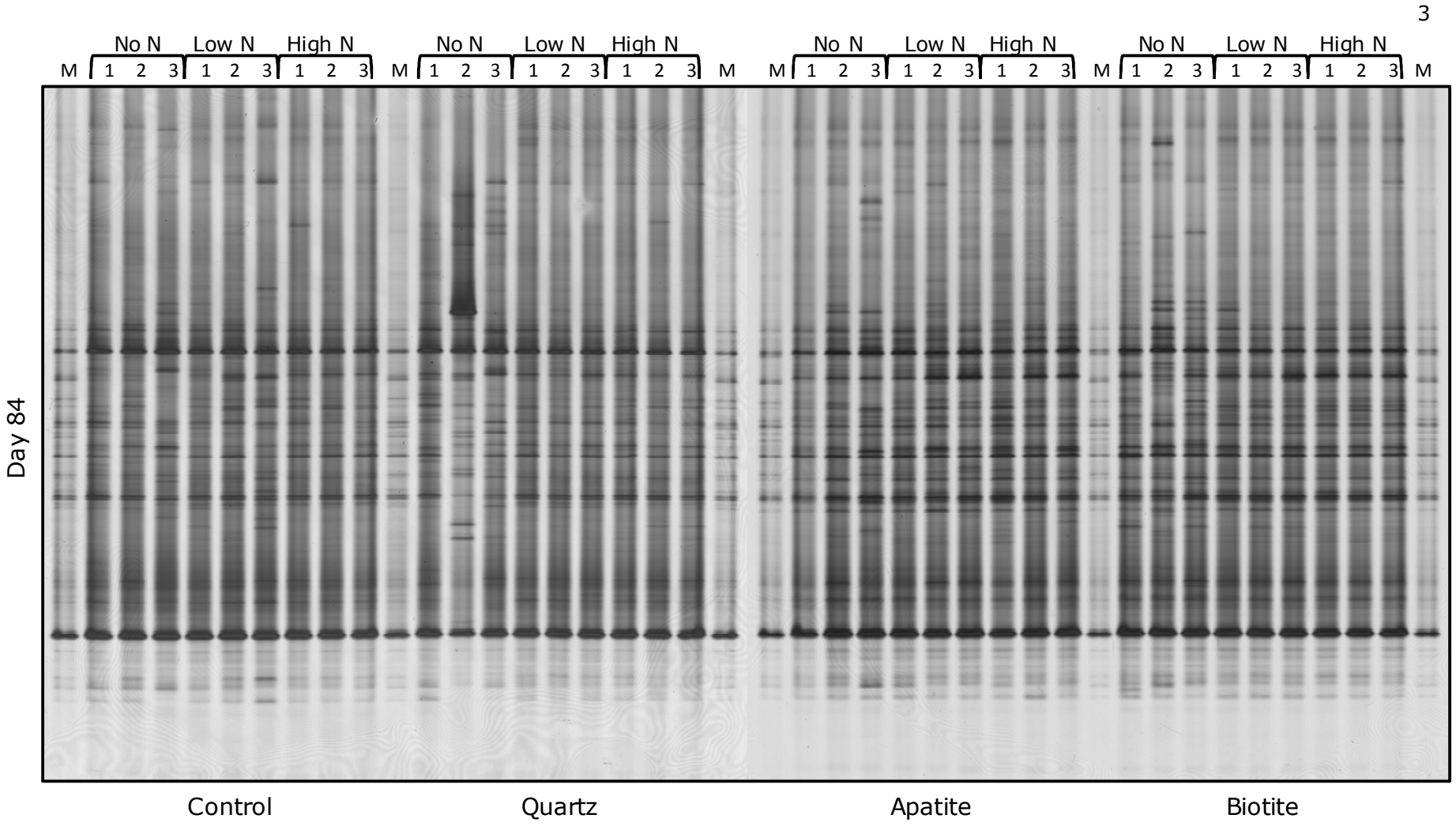
PCR-DGGE GENERAL FUNGAL COMMUNITY PROFILES


1
No N
Low N
High N
M 1 2 3 1 2 3 1 2 3 M
Day 0
Control
Quartz
Apatite
Biotite

2

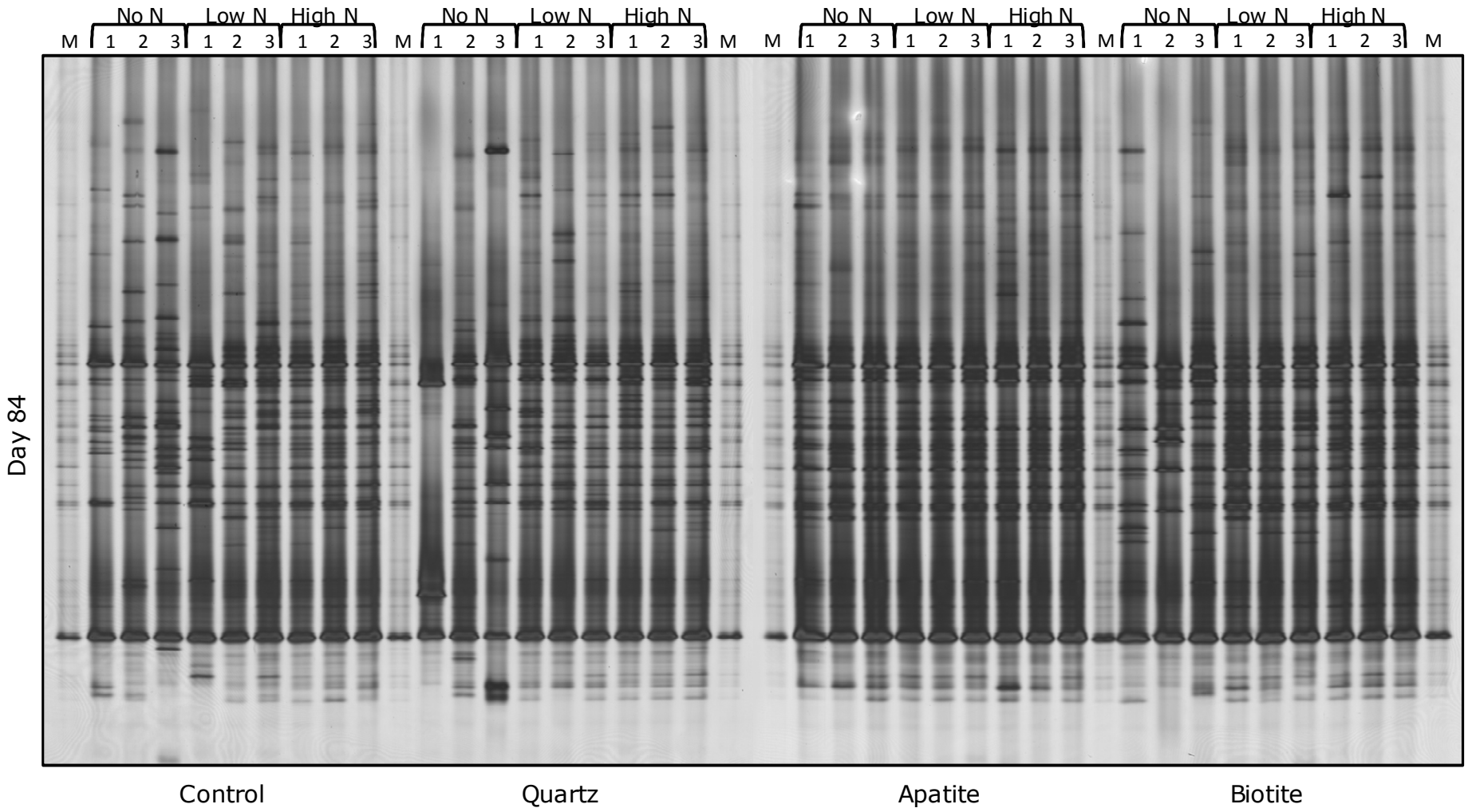


Bulk Soil



Rhizosphere Soil

4



← **Figure A.** Denaturing gradient gel electrophoresis (DGGE) profiles of fungal community based on ITS regions from natural forest soil amended with different N levels (No N, Low N, High N) and primary minerals (Control with no minerals added, Quartz, Apatite, Biotite). Lanes 1-3 in all treatments are samples from triplicate microcosms that were harvested destructively. Markers in lane M consisted in one of the samples from day 0, randomly picked. 1) DGGE profile from soil at day 0, 2) DGGE profile from soil at day 42, 3) DGGE profile from bulk soil at day 84, 4) DGGE profile from rhizosphere soil at day 84.